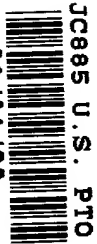


09-22-00

A



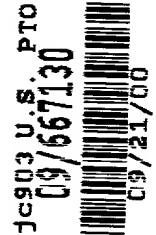
JC885 U.S. PTO

EXPRESS MAIL CERTIFICATE

Date 9/21/00 Label No. E62822274 605

I hereby certify that, on the date indicated above I deposited this paper or fee with the U.S. Postal Service & that it was addressed for delivery to the Assistant Commissioner for Patents, Washington, DC 20231 by "Express Mail Post Office to Addressee" service.

DB Pak [Signature]
Name (Print) Signature



JC903 U.S. PTO

09/21/00

PLEASE CHARGE ANY DEFICIENCY UP TO \$300.00 OR
CREDIT ANY EXCESS IN THE FEES DUE WITH THIS
DOCUMENT TO OUR DEPOSIT ACCOUNT NO. 04-0100

Docket No. 5986/17686-US5

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: John W. BARNWELL

For: PLASMODIUM VIVAX BLOOD STAGE ANTIGENS, ANTIBODIES AND
DIAGNOSTIC ASSAYS

CONTINUING APPLICATION

Hon. Commissioner of
Patents and Trademarks
Washington, DC 20231

Sir:

This is a request for filing under 37 C.F.R. 1.53(b) of a:

☒ Continuation ☐ Divisional

application of the following pending prior application:

Serial No. 08/719,821 Filed: 09/30/96

Of: John W. BARNWELL

For: PLASMODIUM VIVAX BLOOD STAGE ANTIGENS, ANTIBODIES AN
DIAGNOSTIC ASSAYS

Examiner: P. Duffy Group: 1645

09/21/00

1. Enclosed is a copy of the prior application as originally filed (along with a copy of the original Declaration). No amendments identified in the declaration for this prior application introduced new matter. Please use this copy as the application and declaration for the present case.
2. The filing fee is calculated below:

CLAIMS AS FILED, AFTER ACCOMPANYING AMENDMENT

	Claims on File	Number Extra	Rate
Basic Fee			\$690.00
Total Claims	2 - 20 =	0 x \$18	\$0.00
Independent Claims	1 - 3 =	0 x \$78	\$0.00
If Multiple Dependent Claims Are Present, Add \$260.00			\$0.00
Total Filing Fee			\$0.00
For Small Entity (half of preceding total)*			\$

*No. 9 below must be checked to claim this reduction.

3. A check in the amount of \$690 is enclosed.
4. ☐ Cancel claims 1-21.
5. ☒ Amend the specification by inserting before the first line the sentence (check one and fill in):

"This is a ☒ continuation, ☐ division, of application Serial No. 08/719,821 filed September 30, 1996, which is a continuation of application Serial No. 08/478,417, filed June 7, 1995; which is a continuation of application Serial No. 08/072,610 filed June 2, 1993 and issued on July 2, 1996 as U.S. Patent

No. 5,532,133. Each of these prior applications is hereby incorporated herein by reference, in its entirety."

6. ☐ The prior application is assigned to: New York University
7. ☒ A Preliminary Amendment is also enclosed.
8. ☐ Informal Drawings are filed herewith
9. ☐ A verified statement claiming small entity status (check one):
☐ was filed in parent application or ☐ is enclosed.
10. ☐ Priority is claimed from
Country:
Number:
Date:

The priority document

- ☐ was filed in the prior application
☐ is enclosed.

11. The Assignment

- ☒ was recorded at Reel 6694 Frame 0741 on August 19, 1993.
☐ is enclosed.

Respectfully submitted,



Adda C. Gogoris

Reg. No. 29,714

Attorney of Record in Prior Application

DARBY & DARBY P.C.
805 Third Avenue
New York, NY 10022
212-527-7700

CERTIFICATE OF MAILING

I hereby certify that this paper and every paper referred to therein as being enclosed is being deposited with the U.S. Postal Service as first class mail, postage prepaid, in an envelope addressed to: Commissioner of Patents & Trademarks, Washington, DC 20231,

on _____ (Date of Deposit)

Date

Name

EXPRESS MAIL CERTIFICATE

Date 9/21/00 Label No. 628222746 us

I hereby certify that, on the date indicated above I deposited this paper or fee with the U.S. Postal Service & that it was addressed for delivery to the Commissioner of Patents & Trademarks, Washington D.C. 20231 by "Express Mail Post Office to Addressee" service.

Name (Print) D B R C K

Signature [Signature]

Docket No.: 5986/17686-US5

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: John W. Barnwell

Serial No.: Concurrently Herewith Art Unit:

Filed: Concurrently Herewith Examiner:

For: PLASMODIUM VIVAX BLOOD STAGE ANTIGENS, ANTIBODIES AND DIAGNOSTIC ASSAYS

PRELIMINARY AMENDMENT

Hon. Commissioner of
Patents and Trademarks
Washington, DC 20231

Sir:

Prior to the examination of the above-referenced application, please
amend the specification and claims as follows:

In the Specification:

Page 4, line 31, after "sequence" insert -- (SEQ ID NO:1) --.

Page 5, line 18 after "1989." insert as a new paragraph

--EcoRI digest of purified *X. laevis* ribosomal DNA analyzed by electrophoresis on 1% agarose gel. DNA was transferred to a cellulose nitrate strip, which was then cut longitudinally in two. The left-hand side was hybridized to 18 S RNA and the right-hand side to 28 S RNA (spec. act. of RNAs, 1.5×10^6 c.p.m. per μg). Hybridization was done in 1xSSC at 65°C using the vessel shown in Fig. 3. A large excess of cold 28 S RNA was added to the labeled 18 S RNA to compete out any 28 S contamination. After hybridization, the strips were washed in 1 x SSC at 65°C for 1-5 h, and dried. They were then dipped through a solution of PPO in toluene (20%, w/v) dried in air and placed against Kodak RP Royal X-ray film at -70°C for 2 months.--

Page 6, line 32, replace "ATCC, Bethesda, MD" with -- American Type Culture Collection, 10801 University Boulevard, Manassas, VA, 20110-2209 --;

line 33, before "and" insert -- May 26, 1993 --; and
after "No." insert -- HB 11365 --.

Page 7, line 11, replace "ATCC, Bethesda, MD" with -- American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209 --;

line 11, after "on" insert -- May 26, 1993 --;
line 12, after "Nos." insert -- HB 11367 --; and
line 12, after "and" (second occurrence) insert -- HB-11366 --.

Page 8, line 20, after "This sequence" insert -- (SEQ ID NO:3) --; and

line 23, after "EEVEVP" insert -- (SEQ ID NO:4) --.

Page 9, lines 21-22, replace "ATCC, Bethesda, MD." with -- American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209 --;

line 22, after "on" insert -- May 26, 1993 --; and

line 22, after "No." insert -- 69318 --.

In the Abstract:

Line 3, after "directed to" insert -- polynucleotides encoding --.

In the Claims:

Cancel claims 1-21. Please add the following claims:

22. An isolated and purified nucleic acid hybridizable to polynucleotide of SEQ ID NO. 1, under stringent conditions.

23. The isolated and purified nucleic acid of claim 22, wherein said stringent conditions are 1X SSC at 65° C.

REMARKS

The specification has been amended to make the specification consistent with the entered amendments to the specification of the parent application.

Claims 22 and 23 have been added to the present application and claims 1-21 have been canceled. Claim 22 in the present application corresponds to canceled claim 31 in the parent application; claim 23 corresponds to canceled

claim 32.

Claims 22 and 23 (canceled claims 31 and 32 in the parent application) are supported by original claim 5 and the disclosure at page 5, lines 15-18 of the specification, referring to stringent conditions as disclosed by the Southern et al. reference to wash hybridization strips (page 507 and 510 of Southern et al.). Moreover, Applicant incorporated the Southern et al. reference in its entirety in the present application at page 5, lines 9-11. Therefore, the claims are fully supported, no new matter has been added, and the claims are entitled to the priority date of the parent application.

Applicants have also amended the specification at page 5, line 18 to incorporate a specific section of the Southern et al. reference, referring to the hybridization conditions of "1xSSC at 65°C." This amendment is accompanied by a declaration pursuant to MPEP §608.01(p) and *In re Hawkins*, 486 F.2d 569, 179 USPQ 157 (CCPA 1973), *In re Hawkins*, 486 F.2d 579, 179 USPQ 163 (CCPA 1973) and *In re Hawkins*, 486 F.2d 577, 179 USPQ 167 (CCPA 1973). This amendment simply incorporates a portion of the Southern et al. reference that was previously incorporated in its entirety in the parent application. Therefore, this amendment does not constitute new matter, and the amendment is entitled to the priority date of the parent application.

Background

Prior to filing of this divisional application, The Examiner rejected claim 22 (canceled claim 31 in the parent application) in her April 20, 1998 Office Action in the parent application, as being indefinite and vague under 35 U.S.C. §112, second paragraph. In particular, the Examiner stated that the "particular assay conditions are not precisely defined in the specification." In that same Office Action, the Examiner also rejected claim 23 (canceled claim 32 in the parent application) under 35 U.S.C. §112, first paragraph, as containing new matter because the claimed conditions are not explicitly set forth in the passage, and the reference teaching manual (Maniatis) does not define a particular set of conditions which are defined as "stringent."

To further clarify the specification in response to the Examiner's rejections, Applicant amended the specification to include a specific portion of the Southern et al. reference in the Applicant's September 2, 1998 Amendment. The portion previously amended to the specification in the parent application is the same portion of Southern that has been amended to the specification in the present Preliminary Amendment. In the Examiner's September 24, 1998 Advisory Action, the Examiner maintained her previous rejections and refused to enter the amendment, stating that the amendment introduced new matter because Southern et al describes multiple hybridization conditions, none of which were specifically referenced or defined as "stringent" in the reference. Therefore the Examiner stated that "the generic incorporation by reference does not direct one to the particular passage which applicants are relying on for support" and therefore this

passage was new matter.

In support of the amendment of the specification to incorporate a portion of Southern, Applicant submitted an *In re Hawkins* declaration following the Applicant's Notice of Appeal filed October 28, 1998. However, in the Examiner's Office Action mailed November 3, 1999, the Examiner maintained the new matter rejection and stated that *In re Hawkins* was inapplicable to the present situation since *Hawkins* required specific incorporation of page and verse.

The Applicant disagrees with the Examiner's earlier rejections for the reasons previously made of record and for the following reasons.

Rejection of claim 22 for "stringent conditions"

Claim 22 recites an isolated and purified nucleic acid which is hybridizable to SEQ ID NO.1, under stringent conditions. Applicant emphasizes that what is claimed is the hybridizable nucleotide, as opposed to a process limited to specific stringent conditions. In addition, the stringent conditions referred to in claim 22 are fully supported by the specification and fully defined for the following reasons:

First, "stringent conditions" is a term well-known and frequently used in the art. Thus, a person of ordinary skill in 1993 would have no trouble understanding the term nor what is referred to in Southern. As set forth in the Applicant's previous response of January 27, 2000, a cursory search for the terms "stringent or stringency

w/3 condition(s) w/6 hybridization" in U.S. Patents filed before 1994 resulted in 833 patents.

Second, Applicants were entitled to incorporate into the specification the entire Southern reference. The Southern reference is only an article, 18 pages long, and discloses a number of conditions that are stringent. Applicants were entitled by law to incorporate the entire reference, but have not done so in the interest of brevity. If the Examiner wishes, the entire Southern reference can be inserted in the specification.

Third, the contents of the Southern reference are well-known in the art, and Applicants were not required to incorporate them by reference at all. Applicant's attorneys telephoned Scientific Citations and were told that the Southern article has been cited over 20,000 times in the technical and scientific literature. As set forth in the Applicant's response of January 27, 2000, a search of U.S. patents revealed that the Southern article is cited in the specification (not in the list of prior art) in 560 U.S. patents at least 150 of which were already issued in June 1993. Accordingly, the Southern reference can be considered a standard reference in the art and the hybridization conditions set forth in Southern are well-known such that a person skilled in the art would have no trouble understanding that "stringent hybridization conditions" would have meant conditions no less stringent than 1X SCC at 65°C as found in the Southern reference, or the equivalent (higher salt concentration at a lower temperature). Accordingly, those skilled in the art would readily understand what the phrase "stringent conditions" meant as used in the specification and how the phrase

was applied to the Southern article. Thus, the use of the Southern article to illustrate stringent conditions is unambiguous.

Fourth, the phrase “stringent conditions” was never meant to be a direct quotation. As the Applicant has previously noted, the phrase was placed in quotation marks to indicate that the phrase was being defined in the specification and that, when used in the specification, would have the defined meaning. The Examiner statement that the Applicant’s use of the word “by” in the passage “‘Stringent conditions’ are as defined **by** (emphasis added) Southern ...” implies a direct quotation is not supported by any rule of interpretation or case. Applicant’s definition of “stringent conditions” to encompass the hybridization conditions disclosed in Southern is entirely consistent with the use of the word “by” in the quoted passage. Moreover, the defined term “stringent conditions” is one in a list of six defined terms set forth in quotation marks on pp. 5-6 of the specification. There is no reason why the person of ordinary skill would interpret “stringent conditions” to be a direct quotation but all the other terms to be simply defined terms.

Rejection of claim 23 for “1XSSC at 65°C” conditions

Even assuming that the Applicants improperly defined stringent conditions and that Southern does not disclose stringent conditions, then the Applicant's are still entitled to a claim specifying the conditions of “1XSSC at 65°C” as specified in Southern.

First, Applicants were entitled to incorporate into the specification the entire Southern reference. As the Applicant has previously stated, the Southern et al. reference was incorporated in its entirety by the specification on page 5, lines 9-11. Therefore the Applicant was entitled to incorporate the entire Southern reference, but did not do so in the interest of brevity. However, if the Examiner so wishes, the entire Southern reference can be inserted in the specification.

Second, Southern et al. discloses a number of hybridization conditions, including 2X SSC, 6X SSC, 4X SSC, and 1X SSC at the disclosed temperature(s) (See, e.g., Plate II in the Southern reference). Applicant clearly and properly incorporated Southern et al. in its entirety in the original specification. Because Southern et al. specifically discloses a range of conditions, including 1XSSC at 65°C, Applicant's claiming of that particular condition is fully supported in the original specification and does not constitute new matter.

Third, even assuming that Applicant's incorporation of the Southern reference was defective, such defect has been cured by Applicant's use of an *In re Hawkins* declaration pursuant to MPEP §608.01(p), ¶6.19. The Examiner's previous objection to the Applicant's *In re Hawkins* declaration is based on an apparent misreading of the *In re Hawkins* decisions.

According to the Examiner, *In re Hawkins* is distinguishable because *In re Hawkins* "specifically directed one skilled in the art to specific compounds and the methodology used to make them in the identified" references. The Examiner's interpretation of this passage as requiring a specific citation of page and paragraph is

incorrect. In the case of *In re Hawkins*, the court held as sufficiently specific a reference to application serial numbers: "These novel compounds may for example be used in the production of valuable monomers for example by the processes described in copending British applications 36107/66, 42756/66, 46971/66, 49699/66, 50324/66, 10070/67, and 10071/67." *Hawkins*, 179 USPQ at 159. The *In re Hawkins* reference consisted of string cites that were no more specific in terms of pointing to a chapter and verse within each cited reference (and due to their large number could be considered to be even less specific) than Applicant's reference to a single article in the present situation. Therefore the court in *In re Hawkins* held that a later amendment based on these string cites was "not new matter within the meaning of 35 U.S.C. §132 ..." because the information was "identified and specifically referred to for that information in the U.S. application as filed." *Hawkins*, 179 USPQ at 162. Clearly, Applicant's incorporation of the Southern article "in its entirety" meets and exceeds the level of specificity found *In re Hawkins*. Therefore Applicant's *In re Hawkins* declaration is proper and incorporation of the Southern material into the specification is in compliance with MPEP §608.01(p), ¶16.19.

Likewise, notwithstanding the Examiner's assertion otherwise, the case law, including *In re Voss*, 194 USPQ 267 (CCPA 1973) and *In re Fouché*, 169 USPQ 429 (CCPA 1971), support the Applicant's position. The court *In re Voss* held that an earlier rejection, based on *In re Seversky* 474 F.2d 671, 177 USPQ 144 (CCPA 1973), confused two different concepts: "(1) the right to have the benefit of the filing date of an earlier application under [35 USC §] 120 ... and (2) the incorporation by reference in an

application of matter elsewhere written down (not necessarily in a patent application), for economy, amplification, or clarity of exposition ...” *Voss*, 194 USPQ at 270

(emphasis added). In the latter case of the situation where a reference incorporates material only for the purpose of economy, amplification or clarity, the court held that the incorporation of “Reference is made to United States Patent No. 2,920,971, granted to S.D. Stookey, for a general discussion of glass-ceramic materials and their production” was sufficiently specific without need for column and lines to allow later importation of material from the incorporated reference. *Voss*, 194 USPQ at 270.

Similarly, in *In re Fouché*, the court held that “identification was reasonably precise,” even though the “appellant could have used a more precise identification technique ... [and] the technique used does not absolutely distinguish the application sought to be referenced from all other possible applications.” *Fouché*, 169 USPQ at 431 (emphasis added). As in the above cases, Applicant referenced “stringent hybridization” conditions in Southern for the purposes of economy, amplification, or clarity of exposition. Therefore, like *In re Voss* and *In re Fouché*, and unlike a situation where reference is made in order to claim the benefit of an earlier filing date, Applicant in the present situation need not reference to a specific page or paragraph. As a result, *In re Voss* and *In re Fouché* are directly on point and Applicant’s reference to Southern in the present specification has at least the necessary specificity.

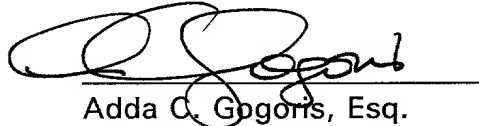
The Examiner also cites a portion of MPEP section 608.01(p), which states “Particular attention should be directed to the specific portions of the referenced

document where the subject matter to be incorporated can be found.” The Examiner states that since the entirety of the reference is cited, it is unclear which portion of the reference Applicant is relying upon. First, Applicant notes that “the Manual of Patent Examining Procedure is merely a guide to the Examiner and is not controlling where it conflicts with the statutes and Rules of Practice.” *Hawkins*, 179 USPQ at 160. A specific reference does not necessarily mean reference to a specific page number or portion. Second, Applicant has identified the referenced background material with sufficient specificity as required by the case law. The decisions in *In re Hawkins*, *In re Voss* and *In re Douche* clearly hold that reference to specific pages or paragraphs is not necessary where material is incorporated for economy, amplification or clarity, and in any event the referenced material here is only one article and is incorporated in its entirety in the specification at p. 5, lines 9-10. The Examiner cites to no case which requires reference to a specific page number or portion for referenced material of reasonable length, such as an article, already incorporated “in its entirety.” Third, reference to a specific portion of the Southern reference is not necessary because the Southern article is extremely well known in the relevant field of biology. As set forth earlier, the Southern reference is an old, well-regarded article that has been cited ample times. The hybridization conditions set forth in Southern are well-known in the field. A person skilled in the art would have no trouble understanding what conditions in Southern Applicant’s phrase “stringent conditions” referred to.

In view of the above remarks, Applicant respectfully submits the claims 2 and 23 are definite, enabled, supported by the specification, and do not constitute new matter. Applicant therefore submits that the claims are in condition for allowance, and a notice to that effect is respectfully requested.

Respectfully submitted,

Dated: September 21, 2000

A handwritten signature in black ink, appearing to read 'Adda C. Gogoris', is written over a horizontal line.

Adda C. Gogoris, Esq.
Reg. No. 29,714
Attorney for Applicant(s)

DARBY & DARBY P.C.
805 Third Avenue
New York, New York 10022
212-527-7700

Date 9/30/96 Label No. EM016 715800
I hereby certify that, on the date indicated above I
deposited this paper or fee with the U.S. Postal Service
& that it was addressed for delivery to the Commissioner
of Patents & Trademarks, Washington, DC 20231 by "Express
Mail Post Office to Addressee" service.

5986/07686

HANIKOTTA HANAN HANIKOTTA HAN
Name (Print) Signature

5

EXPRESS MAIL CERTIFICATE

Date 9 June 1995 Label No. TB45293927
I hereby certify that, on the date indicated above I
deposited this paper or fee with the U.S. Postal Service
& that it was addressed for delivery to the Commissioner
of Patents & Trademarks, Washington, DC 20231 by "Express
Mail Post Office to Addressee" service.

EXPRESS MAIL CERTIFICATE

Date 6/2/93 Label No. TB354506713
I hereby certify that, on the date indicated above I
deposited this paper or fee with the U.S. Postal Service
& that it was addressed for delivery to the Commissioner
of Patents & Trademarks, Washington, DC 20231 by "Express
Mail Post Office to Addressee" service.

HANIKOTTA HANAN HANIKOTTA HAN DR. J. KARASZI
Name (Print) Signature

10

**PLASMODIUM VIVAX BLOOD STAGE ANTIGENS,
ANTIBODIES, AND DIAGNOSTIC ASSAYS**

The U.S. Government has rights in this invention by
virtue of Grant Nos. RO1 AI 24710 from The National Institutes of
Health and DPE-5979-A-00-0006 from the Agency for International
Development.

SUBJECT AREA OF THE INVENTION

This invention is directed to novel species-specific
malarial polypeptides which are secreted into the plasma of a
susceptible mammalian host after infection, and to antibodies
directed against those proteins. The polypeptides and/or anti-
bodies are utilized in assays used to diagnose malaria, as well
as to determine whether *Plasmodium vivax* is the species respon-
sible for the infection.

BACKGROUND OF THE INVENTION

Malaria is transmitted by the bite of the Anopheles
mosquito. Minutes after infection, sporozoites (the mosquito-
hosted stage of the malarial parasite) enter hepatocytes of the
susceptible mammal where they multiply by schizogony and develop
into merozoites. Rupture of the infected cells releases the
merozoites into the blood, where they enter erythrocytes to begin
a phase of asexual reproduction. During acute infections,
malarial parasite protein antigens are known to be released,
accumulate, and circulate in the plasma of infected individuals
(Wilson et al., *The Lancet*, July 26, 1969; Wilson et al., *Inter-
national Journal for Parasitology* 3:511-520, 1973; Wilson et al.,

EXPRESS MAIL CERTIFICATE

Date 9/21/96 Label No. 8662822274 605
I hereby certify that, on the date indicated above I
deposited this paper or fee with the U.S. Postal Service
& that it was addressed for delivery to the Commissioner
of Patents & Trademarks, Washington D.C. 20231 by
"Express Mail Post Office to Addressee" service.

D. Beck
Name (Print)

D. Beck
Signature

Parasitology, 71:183-192; Wilson, *Nature*, 284:451-452, 1980). The release of these antigens of parasitic origin can occur at the time that infected erythrocytes rupture to allow invasive merozoites to invade new red blood cells. The antigens that spill into the host plasma are those that have accumulated in the host cell cytoplasm and internal membranous structures.

Additionally, release of antigen can occur during the intraerythrocytic growth of the parasite as it matures from the ring stage, the stage which invades the erythrocyte, through the trophozoite stage, and into schizogony when the parasite differentiates into merozoites. Release of antigens at this time involves transport of the protein from the parasite across the parasitophorous vacuole and its membrane, across the host cell cytoplasm to the infected erythrocyte membrane, and then secretion as an intact soluble protein into the plasma of the host. One *P. falciparum* protein, PfHRP-2 (Histidine Rich Protein-2) has been described that follows this route of transport and is secreted into the culture supernatant or found in plasma (Wellems et al., *Proc. Natl. Acad. Sci. USA*, 83:6065-6069, 1986; Howard et al., *J. of Cell. Biol.*, 103:1269-1277, 1986; Rock et al., *Parasitology*, 95:209-227, 1987; Panton et al., *Mol. and Biochem. Parasitology*, 35:149-160, 1989). A search for HRP analogues in *P. vivax* using PfHRP gene probes and HRP-antisera gave only negative results (Rock et al., *Parasitology*, 95:209-227, 1987; J. Barnwell, unpublished results).

There is a need in the field for antibodies specific for a *P. vivax* blood stage protein in a diagnostic assay. The prior art assays based on antibodies specific for blood stage proteins have been specific only for *P. falciparum* (Khusmith, *Southeast Asian J Trop Med Public Health (THAILAND)*, 19:21-6, 1988) or have involved the use of panspecies-specific antibodies, so no existing assays are specific for *P. vivax* (Gao et al., *Southeast Asian J Trop Med Public Health (THAILAND)*, 22:393-6, 1991 and James, MA et al., American Society of Tropical Medicine and Hygiene, Seattle, WA, Nov. 16-19, 1992, Abs. #135, pp. 145-

146). *P. vivax* has latent liver stages, termed hypnozoites, which are reactivated and reinitiate blood stage parasitemias. Hypnozoites are eliminated by treatment with primaquine, but are not affected by chloroquine, which acts only on blood stage parasites. As *P. falciparum* does not produce hypnozoites, it is important to identify correctly the *Plasmodium* species responsible for infection in order to provide the appropriate course of chemotherapy for complete cure. The increased prevalence of drug resistant strains in certain species also makes it important to identify the species involved so correct chemotherapy can be given. Thus, there is a need for a method and reagents adapted for differential diagnosis of *P. vivax* malaria.

However, a number of criteria should be met by a particular protein antigen considered as a potential diagnostic target. First, it should be soluble and relatively stable and not rapidly degraded and/or rapidly removed from circulation. Second, the antigen should contain epitopes unique to a species to allow specific diagnosis and preferably be well-conserved within all or most isolates of a species. Additionally, it should be relatively abundant to allow detection at low parasitemia. As discussed below, the proteins of this invention fulfill most or all of these requirements.

SUMMARY OF THE INVENTION

Secreted species-specific blood stage antigens have now been identified from a major human malaria parasite species, *P. vivax*. Two particular such proteins are designated *P. vivax* Erythrocyte Secreted Protein-1 (PvESP-1) and *P. vivax* Erythrocyte Secreted Protein-2 (PvESP-2). These antigens and fragments thereof have unique *P. vivax*-specific epitopes which permits their use in differential determination of *P. vivax* merozoites. Antibodies can be and have been elicited against unique epitopes of such *P. vivax* proteins and used in assays which not only diagnose malaria, but also selectively identify *P. vivax* as the species having caused the infection.

DESCRIPTION OF THE DRAWINGS

Figs. 1A and 1B are Western immunoblots of *P. vivax* trophozoite infected erythrocytes probed with antibodies specific for PvESP-1 and PvESP-2. They show that mAb 1D11.G10 reacts with
5 a 225 KD protein, while mAb 3D4.E2 and 1A3.B4 react with a 70 KD protein.

Figs. 2A and 2B are Western immunoblots of *P. vivax* infected erythrocytes and supernatant from cultures which were matured from ring stage to late-staged trophozoites *in vitro*.
10 The blots are probed with mAbs specific for PvESP-1 (2A) and PvESP-2 (2B). They show that both PvESP-1 and PvESP-2 are present in isolated infected erythrocytes and in the culture medium.

Fig. 3A is a schematic representation of the *P. vivax* ESP-1 gene and structural features of the deduced protein. Fig.
15 3B is a partial restriction map of the *P. vivax* ESP-2 gene.

Fig. 4A is an immunoblot of *P. vivax* culture supernatants and plasma from *P. vivax* infected squirrel (*Saimiri*) monkeys. 4B is an immunoblot of multiple species of *Plasmodium*
20 in multiple stages probed with PvESP-1 specific antibodies. Figs. 4C and 4D are immunoblots of plasma from individuals infected with *P. falciparum*, *P. vivax* or both, and also probed with PvESP-1 specific antibodies. This group of figures shows the selective reaction of these antibodies with *P. vivax* and with
25 proteins in the plasma of those infected with *P. vivax*. Similar results can be obtained with PvESP-2 antibodies using immunoblot procedures. (Example 5) Similar results for malaria specificity are also obtained for PvESP-1 or PvESP-2 antibodies on smears of different species of malaria parasites by indirect immunofluores-
30 cence assay.

Fig. 5 is the DNA sequence and deduced amino acid sequence of *P. vivax* ESP-1 (a sequence listing is provided separately).

Figs. 6A and B are the immunoelectron micrographs of *P. vivax* infected erythrocytes probed with mAb 1D11.G10 and mAb 3D4.A2, respectively.

Figs. 7A and B are immunofluorescent assays of *P. vivax* infected erythrocytes reacted with fluorescence-conjugated mAb 1D11.G10 and mAb 3D4.A2, respectively.

DETAILED DESCRIPTION OF THE INVENTION

All U.S. patents and references referred to herein are hereby incorporated by reference in their entirety. In case of conflict, the present disclosure controls.

The following definitions apply to the terms as used in this application only and should not be construed to necessarily apply to uses of the terms in other art.

"Stringent conditions" are as defined by Southern et al. in *J. of Mol. Bio.*, 98:503 and as detailed in Maniatis, *Molecular Cloning: A Laboratory Manual*, 2nd ed., Chapter 9, 1989.

"Immunoreactive fragment" means a fragment of an antigen that is recognized by an antibody raised against the entire antigen.

"Immunoreactive analog" means a polypeptide which differs from a naturally occurring or recombinant protein by the substitution, deletion and/or addition of one or more amino acids but which retains the ability to be recognized by an antibody raised against the entire protein. A nonlimiting example is a carrier/antigen fusion polypeptide of the whole antigen or an immunoreactive fragment thereof, where the antigen or fragment can be embedded within the carrier polypeptide or linked to the carrier polypeptide at either end.

"Detecting" means determining the presence (or absence) or quantity of a substance (e.g. an antigen-antibody complex).

"Antibody" includes intact antibody molecule or fragments thereof that recognize antigen (e.g. Fab or F(ab')₂ fragments) and can be of polyclonal or monoclonal type.

"Epitope" means any antigenic determinant responsible for immunochemical binding with an antibody molecule. Epitopes usually reside within chemically active surface groupings of protein molecules (including amino acids and often also sugar side-chains) and have specific three-dimensional structural characteristics and specific charge characteristics.

"Peptide Antigen" means a peptide, dipeptide, or polypeptide that can elicit (or react with) antibodies recognizing a particular protein.

The search for secreted blood stage antigens for *P. vivax* began by making monoclonal antibodies specific for blood stage parasites. As described in detail in Example 1, the mAbs were made by conventional techniques through the fusion of spleen cells isolated from a mouse immunized with *P. vivax* infected red blood cells with mouse myeloma cells to produce mAb secreting hybridomas. Three of these mAbs were found to react with the *P. vivax* proteins described herein. These proteins have been shown to be synthesized by the parasite by several criteria. First, mAbs do not react with uninfected erythrocytes as shown by control experiments and the specificity of the mAb for *P. vivax*, described in Example 6. A reaction to all species would be seen if the proteins were erythrocytic. Second, as seen by IFA and IEM, the mAbs do not react with uninfected erythrocytes which are present in the preparations. (See Figures 6 and 7) Third, the mAbs have been used to immunoprecipitate radiolabelled proteins from extracts of parasites that have been biosynthetically labelled with ³⁵S-methionine. These results indicate that the mAbs recognize *P. vivax* proteins.

Specifically, mAb 1D11.G10 recognizes a *P. vivax* protein of approximately 225,000 daltons in size as judged by SDS-PAGE (Fig. 1A, lane 1). The hybridoma which produces mAb 1D11.G10 has been deposited with the ATCC, Bethesda, MD, on _____ and is Accession.No. _____. The protein recognized by this mAb has been designated *P. vivax* ESP-1 or PvESP-1. It is found in the culture supernatant when intact infected eryth-

rocytes are incubated *in vitro* for 10-24 hours (Fig. 2A, lane labelled SUP) as well as supernatant collected from *in vitro* cultures of rupturing mature schizont infected red cells (lane labelled IRBC). These data indicate that this protein is
5 secreted. It is localized by immunofluorescent assay (IFA) and immuno-electron microscopy (IEM) to the erythrocyte membrane of infected erythrocytes. (Figures 6A and 7A)

mAbs 3D4.A2 and 1A3.B4 recognize a *P. vivax* protein of approximately 70,000 daltons in size as judged by SDS-PAGE (Fig.
10 1B, lane 1). The hybridomas which produces mAbs 3D4.A2 and 1A3.B4 have been deposited with the ATCC, Bethesda, MD, on _____ and are Accession Nos. _____ and _____, respectively. The protein recognized by these mAb has been designated *P. vivax* ESP-2 or PvESP-2. Like PvESP-1, PvESP-2 is found in the
15 supernatants of *in vitro* cultured intact trophozoite-infected erythrocytes (Fig. 2B, lane labelled SUP), and thus is a secreted protein. The PvESP-2 protein is also found in culture supernatants collected after schizont-infected erythrocytes have ruptured and released merozoites (lane labelled IRBC). The
20 protein is localized by IFA and IEM to the caveola-vesicle complexes (CVC) of *P. vivax* infected erythrocytes. (Figures 6B and 7B.) The CVC are membranous sac-like vesicles attached to and contiguous with areas of flask-shaped indentations called caveolae in the erythrocyte plasma membrane.

25 Polyclonal antibodies can also be produced, for example, using isolated PvESP-1 and/or PvESP-2 or fragments thereof. General methodology for making such polyclonal antibodies is well-known in the art, and can be made using protocols similar to that described in Pink et al. (*Eur. J.*
30 *Immunol.* 4:426-429, 1974).

The mAbs described above were used to screen a λ ZAP recombinant phage library of the *P. vivax* genome, although other equivalent *P. vivax* libraries could have been used. The preparation of this library is described in Example 2. After induc-
35 tion, mAb 1D11.G10 specifically recognized one plaque, designated

PvMB3.3.1. The 3.34 kB plasmid insert was isolated and sequenced. The resulting sequence is SEQ ID No: 1. The nucleic acid sequence was analyzed for open reading frames (ORF) and the deduced amino acid sequence of the encoded protein was determined. The amino acid sequence is SEQ ID No: 2. A schematic structure of the gene and features of the encoded protein is presented in Fig. 3A. The gene appears to be missing a small portion of its 5' end.

As shown in Figs. 3A and 5, the deduced amino acid sequence has an initial (N-terminal) sequence of hydrophobic amino acids. This is followed by a short 139 base pair (bp) intron with typical malaria intervening sequence splice sites. There follows a 2964 bp ORF, ending in the TAA stop codon which is 53 bp before the end of the cloned 3.34 kB insert DNA. A protein having this deduced peptide sequence is hydrophilic with a low pI (3), consistent with a large proportion of glutamate (Glu or E) residues in the deduced amino acid sequence.

As indicated in the Figures, there are two sets of repeated amino acid units in the sequence. One repeat unit is characterized by the sequence D(L/M)EAGEE(A/T)G. This sequence is repeated 7 times at the N-terminal end of the protein. The second repeat is located in the C-terminal portion of the protein, has the sequence EEVEEVP, and is repeated 10 times. The hydrophobic amino acid sequence could potentially be, as judged by its computer analyzed hydrophobicity profile, a transmembrane domain, or a leader or signal peptide sequence, or act as both. Completion of the 5' gene sequence will shed more light on these possibilities, and is well within the skill of the art in light of the present disclosure.

To determine the remainder of the gene sequence, the complete intact gene can be isolated and sequenced using a large DNA fragment, for example, in a Lambda replacement vector such as Lambda DASH (Stratagene, LaJolla, CA) or equivalent library using the insert as a probe. Methodology for this is provided by Galinski et al. (Cell, 69:1213-1226, 1992) or other similar

methods. Alternatively, the 5' end could be isolated by the PCR amplification or other method of amplification of the cDNA using appropriate primers, for example, as described by Frohman et al. (*Proc. Natl. Acad. Sci. USA*, 85:8998-9002, 1988).

5 The MB3.3.1 plasmid expresses in *E. coli* a large recombinant protein recognized by the mAb 11D.G10 in Western immunoblots. The topmost band recognized is approximately 205-210 Kd in size, confirming that a small portion of the complete PvESP-1 gene remains unsequenced since the native protein
10 migrates in SDS-PAGE at 225 Kd under identical conditions. This protein is easily isolated from the culture using well-known techniques (Maniatis, *Molecular Cloning: A Laboratory Manual*, 2nd ed., Chapter 18, 1989). Mouse and rabbit antisera generated by immunization with the 1D11.G10 affinity purified recombinant
15 protein recognizes both the recombinant and native PvESP-1 indicating that the recombinant phagmid MB3.3.1 authentically encodes PvESP-1.

Screening of the λ ZAPII expression libraries with the mAB 3D4.E2 revealed one phage plaque recognized by antibody.
20 This clone, PvMB2.5.1, was found to contain a plasmid having a 3.7 kB insert. This plasmid has been deposited with the ATCC, Bethesda, MD. on _____ having Accession No. _____. A partial restriction map of the plasmid insert is depicted in Fig. 3B. The sequence of this DNA is easily obtainable using, e.g.,
25 traditional nested deletion and subcloning techniques or using nucleic acid primers obtained by dideoxy sequencing of the insert (Maniatis, *Molecular Cloning: A Laboratory Manual*, 2nd ed., Chapter 13, 1989).

30 The MB2.5.1 plasmid expresses a recombinant polypeptide of approximately 60 KD, which is easily isolated from the culture medium using standard protein isolation techniques (Maniatis, *Molecular Cloning: A Laboratory Manual*, 2nd ed., Chapter 18, 1989). The size of this protein suggests that a portion of the coding region of this protein is not present in the insert, as
35 the native protein migrates under identical conditions in SDS-

PAGE at approximately 70 KD. The 60 KD polypeptide is recognized by mAb 3D4.E2 in Western blots, indicating the recognized epitope is encoded by the insert. Additionally confirmation that plasmid MB 2.5.1 authentically encodes PvESP-2 can be done as for PvESP-1, by immunizing with recombinantly expressed antigen and using the antisera to determine if it recognizes native 70 kD PvESP-2.

Both PvESP-1 and PvESP-2 are successfully expressed in *E. coli* and expression of these proteins in other systems, such as viral systems, other bacterial systems, yeast systems or mammalian cell culture, is also contemplated and is well within the skill of the art. Using such alternate expression systems may be preferred if glycosylation or other post-translational modification is desired.

Purified native or recombinant peptide antigens of the present invention can be used in immunogenic preparation to raise additional antibodies. Such preparations will include immunogenically effective amounts of the present antigens as well as pharmaceutically acceptable vehicles, carriers, buffers, fillers adjuvants and/or diluents.

Peptide antigens of the present invention can be purified by well-known chromatographic techniques. Examples include SDS or native PAGE gel elution, size exclusion gel filtration, anion/cation exchange, antibody affinity, protein binding to immobilized glutathione or MBP fusion partner binding to immobilized amylase, metal binding with a polyhistadine linker peptide after expression in a suitable plasmid vector and cell host, or combinations thereof.

As is evident to one of ordinary skill, it is only certain portions or epitopes of the proteins which are recognized by Mabs. The portions of the protein(s) containing the relevant epitopes can be identified. Fragments of the gene can be subcloned into the appropriate reading frame of a plasmid expression vector and used to transfect *E. coli* (or other host system) and expressed by induction. Defined gene fragments can be generated using restriction enzymes with cutting sites within

the gene. Alternatively, appropriate oligonucleotide primers can be used in a PCR-based amplification reaction to engineer the DNA fragment to be subcloned into the plasmid expression vector. Once the expression vector is constructed, the recombinant immunoreactive fragments (or analogs, e.g. as a fusion polypeptide) are expressed. The produced fragments are then reacted with antibodies as above in Western immunoblots, ELISA tests, or other immunochemical assay methods to determine which portion or portions of the protein specifically interact with the antibodies. These methods work well for defining relatively large or small regions of a protein to locate the corresponding epitope and is effective in identifying conformation-dependent (discontinuous) epitopes, or linear epitopes. An alternative method for identifying antigenic determinants is the use of overlapping synthetic peptides of 8-15 amino acids that correspond to the deduced amino acid sequence of the gene. Reactivity of these peptides can be determined using ELISA-based assays, such as the methodology of Geysen et al. (*Journal of Immunological Methods*, 102:259-274, 1987) or using commercial-based peptide synthesis kits, i.e., Pepscan or Inimotope. (Cambridge Research Biochemicals, Valley stream, NY and Chiron, Emeryville, CA, respectively) This method is especially effective in determination of linear epitopes.

The detection of parasite antigens present in a biological fluid (e.g. plasma), such as PvESP-1 and PvESP-2, can constitute a method for the diagnosis of acute or chronic *P. vivax* malaria infections. To be useful, such an antigen should contain epitopes unique to the *P. vivax* species to allow specific diagnosis and differential diagnosis from other malarial infections, and should preferably be conserved within all or most isolates of that species (more than one antigens can be used to generate antibodies if necessary to accommodate strain variations). Either monoclonal antibodies or polyclonal antibodies could be used in the assay, with monoclonals preferred. The epitopes recognized by the monoclonal antibodies 1D11.G10 (anti-

PvESP-1) and 3D4.E2 (anti-PvESP-2) are present in all or most *P. vivax* so far tested (25/26 for 1D11.G10 and 26/26 for 3D4.E2). However, these antigens are not present in *P. falciparum* (Fig. 4A, lane 3), *P. malariae* (lane 2), *P. coatneyi* (lane 4), *P. knowlesi* (lane 5), or *P. berghei* (lane 1). 1D11.G10 does cross-react with *P. cynomolgi* (lane 6), a simian malaria parasite very closely related to *P. vivax* (lane 7), but never found to occur as a naturally acquired human malaria infection. The mAB 3D4.E2 in particular only recognizes *P. vivax* and thus far does so 100% of the time. Of course, any strain differences that may be encountered may be accounted for in an assay by provision of additional appropriate antibodies, or by provision of antibodies directed to inter-strain conserved epitopes, which can be conveniently raised against recombinant versions of PvESP-1 and PvESP-2 as well as immunoreactive fragments and analogs thereof.

The detected antigens are relatively stable *in vivo*; that is, they are not rapidly degraded and/or removed from circulation. PvESP-1 and PvESP-2 can be detected by Western immunoblot in the plasma of squirrel (*Saimiri*) monkeys experimentally infected with *P. vivax* (Fig. 4A, lane 3) and in the plasma of humans from endemic areas that are infected with *P. vivax* (Figs. 4C, lanes 8-11 and 4D, lanes 5-7). The antigens are not detected in plasma of individuals infected only with *P. falciparum* (Figs. 4C, lanes 3-7 and 4D, lanes 6 and 7), the major human malaria parasite that must be differentiated from *P. vivax*. The squirrel monkey model closely approximates what would occur in naturally infected humans, but under more controlled conditions than that of work conducted in the field within endemic areas. In *Saimiri* monkey infections, the antigen can be detected with the present antibodies when there are 1000 parasites/ μ l blood. In humans, early acute infections are detected. Again, as is evident to one of ordinary skill, the isolation of the genes means that high-titer, high-affinity (e.g., of the order of 10^{10} liters/mol) antibodies can be produced using standard

methodology. These antibodies will be used to increase the sensitivity and specificity of the assay.

Other serological assay formats based on antigen capture and a reporter signal have produced similar results as described above using mABs 3D4.1E2 and 1D11.G10. Based on these successes, it is anticipated that these mAbs or others to be produced using the recombinant proteins or immunogenic fragments thereof can be adapted for use in immunoassay systems (using either labelled Abs or labelled antigens) well-known in the diagnostic testing art.

All well-known methods of labelling antibodies are contemplated, including without limitation enzymatic conjugates, direct labelling with dye, radioisotopes, fluorescence, or particulate labels, such as liposome, latex, polystyrene, and colloid metals or nonmetals. Multiple antibody assay systems, such as antigen capture sandwich assays, are also within the scope of this invention. Further, competitive immunoassays involving labelled protein or assays using the labelled protein to detect serum antibodies are also contemplated forms of the diagnostic assays of the present invention. Beyond diagnostic assays which occur in solution, assays which involve immobilized antibody or protein are also considered within the scope of the invention. (See, for example, Miles et al., *Lancet* 2:492, 1968; Berry et al., *J. Virol. Met.* 34:91-100, 1991; Engvall et al., *G. Immunochemistry*, 8:871, 1971, Tom, *Liposomes and Immunology*, Elsevier/ North Holland, New York, New York, 1980; Gribnau et al., *J. of Chromatogr.* 376:175-89, 1986 and all references cited therein).

Examples of the types of labels which can be used in the present invention include, but are not limited to, enzymes, radioisotopes, fluorescent compounds, chemiluminescent compounds, bioluminescent compounds, particulates, and metal chelates. Those of ordinary skill in the art will know of other suitable labels for binding to the monoclonal or polyclonal antibody (or to an antigen) or will be able to ascertain the same by the use of

routine experimentation. Furthermore, the binding of these labels to the monoclonal or polyclonal antibody (or antigen) can be accomplished using standard techniques commonly known to those of ordinary skill in the art.

5 One of the ways in which an assay reagent (generally, a monoclonal antibody, polyclonal antibody or antigen) of the present invention can be detectably labeled is by linking the monoclonal antibody, polyclonal antibody, or antigen to an enzyme. This enzyme, in turn, when later exposed to its sub-
10 strate, will react with the substrate in such a manner as to produce a chemical moiety which can be detected as, for example, by spectrophotometric or fluorometric means.

Examples of enzymes which can be used to detectably label the reagents of the present invention include malate
15 dehydrogenase, staphylococcal nuclease, delta-V-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-VI-phosphate
20 dehydrogenase, glucoamylase and acetylcholine esterase.

The presence of the detectably labeled reagent of the present invention can also be detected by labeling the reagent with a radioactive isotope which can then be determined by such means as the use of a gamma counter or a scintillation counter.
25 Isotopes which are particularly useful for the purpose of the present invention are ^3H , ^{125}I , ^{32}P , ^{35}S , ^{14}C , ^{51}Cr , ^{36}Cl , ^{57}Co , ^{58}Co , ^{59}Fe and ^{75}Se .

It is also possible to detect the binding of the detectably labeled reagent of the present invention by labeling
30 the monoclonal or polyclonal antibody with a fluorescent compound. When the fluorescently labeled reagent is exposed to light of the proper wave length, its presence can then be detected due to the fluorescence of the dye. Among the most commonly used fluorescent labelling compounds are fluorescein

isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

The reagents according to the invention also can be detectably labeled using fluorescent emitting metals such as ¹⁵²Eu, or others of the lanthanide series. These metals can be attached to the reagent molecule using such metal chelating groups as diethylenetriaminepentaacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA) and salts thereof.

The reagents of the present invention also can be detectably labeled by coupling it to a chemiluminescent compound. The presence of the chemiluminescent-tagged reagent is then determined by detecting the presence of luminescence that arises during the course of the chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester.

Likewise, a bioluminescent compound may be used to label the reagent of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent reagent is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

Another technique which may also result in greater sensitivity when used in conjunction with the present invention consists of coupling the monoclonal or polyclonal antibody of the present invention to low molecular weight haptens. The haptens can then be specifically detected by means of a second reaction. For example, it is common to use such haptens as biotin (reacting with avidin) or dinitrophenol, pyridoxal and fluorescamine (reacting with specific antihapten antibodies) in this manner.

Any biological sample containing the detectable yet unknown amount of *P. vivax* specific blood-stage antigen can be used to assay. Normally, the sample is preferably a liquid, such

as, for example, urine, saliva, cerebrospinal fluid, blood, serum and the like, or a solid or semi-solid, such as, for example, tissue, feces and the like.

It appears that 1D11.G10 may recognize a repeated epitope since it has been successfully used in a two-site antigen capture immunoassay using the same mAb for capture and an alkaline phosphatase labelled mAb or mAb conjugated to liposomes encapsulating a marker dye for the reporter antibody (Example 6). Eleven of 15 plasma samples from *P. vivax* infected individuals were positive by alkaline phosphatase conjugated antibody. Thirteen of 15 samples were positive by liposome conjugated antibody. None of 18 *P. falciparum* infected plasma samples were positive. Therefore, antibodies to PvESP-1 and PvESP-2 appear quite effective when used in a diagnostic assay of *P. vivax* infection and further, such assays appear to specifically identify *P. vivax* infection. It is anticipated that assays based on mAb specific for particular epitopes and selected for their high titer and/or affinity will serve to increase the specificity and sensitivity of the assay.

In general, increases in sensitivity are a development consideration and are achieved by optimization of all reagents, including the concentrations conjugated to reporter systems, adsorbed to solid phase surfaces, specificity of the Abs, and affinity of the Abs. These steps are routinely done and evaluated during assay development and are well within the skill of those working in the art.

As is evident to one of ordinary skill, the diagnostic assay of the present invention includes kit forms of such an assay. This kit would include anti-PvESP-1 and/or anti-PvESP-2 monoclonal or polyclonal antibodies (raised against whole PvESP or immunoreactive fragments or analogs thereof) which can be optionally immobilized, as well as any necessary reagents and equipment to prepare the biological sample for and to conduct analysis, e.g. preservatives, reaction media such as nontoxic buffers, microtiter plates, micropipettes, etc. The reagent (Abs

and/or antigens) can be lyophilized or cryopreserved. As described above, depending on the assay format, the antibodies can be labelled, or the kit can further comprise labelled PvESP-1 or PvESP-2 protein or fragments or analogs thereof containing the relevant epitopes.

The types of immunoassays which can be incorporated in kit form are many. Typical examples of some of the immunoassays which can utilize the antibodies of the invention are radioimmunoassays (RIA) and immunometric, or sandwich, immunoassays.

"Immunometric assay" or "sandwich immunoassay", includes simultaneous sandwich, forward sandwich and reverse sandwich immunoassays. These terms are well understood by those skilled in the art. Those of skill will also appreciate that the monoclonal antibodies, polyclonal antibodies and/or antigens of the present invention will be useful in other variations and forms of immunoassays which are presently known or which may be developed in the future. These are intended to be included within the scope of the present invention.

In a forward sandwich immunoassay, a sample is first incubated with a solid phase immunoabsorbent containing monoclonal or polyclonal antibody(ies) against the antigen. Incubation is continued for a period of time sufficient to allow the antigen in the sample to bind to the immobilized antibody in the solid phase. After the first incubation, the solid phase immunoabsorbent is separated from the incubation mixture and washed to remove excess antigen and other interfering substances, such as non-specific binding proteins, which also may be present in the sample. Solid phase immunoabsorbent containing antigen bound to the immobilized antibody is subsequently incubated for a second time with soluble labeled antibody or antibodies. After the second incubation, another wash is performed to remove unbound labeled antibody(ies) from the solid phase immunoabsorbent and removing non-specifically bound labeled antibody(ies). Labeled antibody(ies) bound to the solid phase immunoabsorbent is then detected and the amount of labeled antibody detected serves as a

direct measure of the amount of antigen present in the original sample.

Alternatively, labeled antibody which is not associated with the immunoabsorbent complex can also be detected, in which case the measure is in inverse proportion to the amount of antigen present in the sample. Forward sandwich assays are described, for example, in U.S. Pat. Nos. 3,867,517; 4,012,294 and 4,376,110.

In carrying out forward immunometric assays, the process may comprise, in more detail: (a) first forming a mixture of the sample with the solid phase bound antibody(ies) and incubating the mixture for a time and under conditions sufficient to allow antigen in the sample to bind to the solid phase bound antibody(ies), (b) adding to the mixture after said incubation of step (a) the detectably labeled antibody or antibodies and incubating the new resulting mixture for a time and under conditions sufficient to allow the labeled antibody to bind to the antigen-antibody complex on the solid phase immunoabsorbent; (c) separating the solid phase immunoabsorbent from the mixture after the incubation in step (b); and (d) detecting either the labeled antibody or antibodies bound to the antigen-antibody complex on the solid phase immunoabsorbent or detecting the antibody not associated therewith.

In a reverse sandwich assay, the sample is initially incubated with labeled antibody(ies), after which the solid phase immunoabsorbent containing multiple immobilized antibodies is added thereto, and a second incubation is carried out. The initial washing step of a forward sandwich assay is not required, although a wash is performed after the second incubation. Reverse sandwich assays have been described, for example, in U.S. Pat. Nos. 4,098,876 and 4,376,110.

In carrying out reverse immunometric assays, the process may comprise, in more detail; (a) first forming a mixture of the sample with the soluble detectably labeled antibody for a time and under conditions sufficient to allow antigen in the

sample to bind to the labeled antibody; (b) adding to the mixture after the incubation of step (a) the solid phase bound antibodies and incubating the new resulting mixture for a time and under conditions sufficient to allow antigen bound to the labeled antibody to bind to the solid phase antibodies; (c) separating the solid phase immunoadsorbent from the incubating mixture after the incubation in step (b); and (d) detecting either the labeled antibody bound to the solid phase immunoadsorbent or detecting the labeled antibody not associated therewith.

In a simultaneous sandwich assay, the sample, the immunoadsorbent having multiple immobilized antibodies thereon and labeled soluble antibody or antibodies are incubated simultaneously in one incubation step. The simultaneous assay requires only a single incubation and does not include washing steps. The use of a simultaneous assay is by far the preferred one. This type of assay brings about ease of handling, homogeneity, reproducibility, and linearity of the assays and high precision. The sample containing antigen, solid phase immunoadsorbent with immobilized antibodies and labeled soluble antibody or antibodies is incubated under conditions and for a period of time sufficient to allow antigen to bind to the immobilized antibodies and to the soluble antibody(ies). In general, it is desirable to provide incubation conditions sufficient to bind as much antigen as possible, since this maximizes the binding of labeled antibody to the solid phase, thereby increasing the signal. Typical conditions of time and temperature are two hours at 45°C, or twelve hours at 37°C. Antigen typically binds to labeled antibody more rapidly than to immobilized antibody, since the former is in solution whereas the latter is bound to the solid phase support. Because of this, labeled antibody may be employed in a lower concentration than immobilized antibody, and it is also preferable to employ a high specific activity for labeled antibody. For example, labeled antibody might be employed at a concentration of about 1-50 ng per assay, whereas immobilized antibody might have a concentration of 10-500 ng per

assay per antibody. The labeled antibody might have a specific activity with, for instance, one radioiodine per molecule, or as high as two or more radioiodines per molecule of antibody.

Of course, the specific concentrations of labeled and immobilized antibodies, the temperature and time of incubation as well as other assay conditions can be varied, depending on various factors including the concentration of antigen in the sample, the nature of the sample and the like. Those skilled in the art will be able to determine operative and optimal assay conditions for each determination by employing routine experimentation.

After the single incubation period, the solid phase immunoabsorbent is removed from the incubation mixture. This can be accomplished by any of the known separation techniques, such as sedimentation and centrifugation. A washing step is not required prior to detection of bound labeled antibody. Detection can be performed by a scintillation counter, for example, if the label is a radioactive gamma-emitter, or by a fluorometer, for example, if the label is a fluorescent material. In the case of an enzyme label, the detection can be done by calorimetric methods employing a substrate for the enzyme.

In carrying out the simultaneous immunometric assay on a sample containing a multivalent antigen, the process may comprise, in more detail:

(a) simultaneously forming a mixture comprising the sample, together with the solid phase bound antibody and the soluble labeled antibody or antibodies;

(b) incubating the mixture formed in step (a) for a time and under conditions sufficient to allow antigen in the sample to bind to both immobilized and labeled antibodies;

(c) separating the solid phase immunoabsorbent from the incubation mixture after the incubation; and

(d) detecting either labeled antibody bound to the solid phase immunoabsorbent or detecting labeled antibody not associated therewith.

Other such steps as washing, stirring, shaking filtering and the like may of course be added to the assays, as is the custom or necessity for any particular situation.

5 In the preferred mode for performing the assays it is important that certain "blockers" be present in the incubation medium (usually added with the labeled soluble antibody). The "blockers" are added to assure that non-specific proteins, protease, or human antibodies to mouse immunoglobulins present in the experimental sample do not cross-link or destroy the
10 monoclonal or polyclonal antibodies on the solid phase support, or the radiolabeled indicator antibody, to yield false positive or false negative results. The selection of "blockers" therefore adds substantially to the specificity of the assays described in the present invention.

15 It has been found that a number of nonrelevant (i.e., nonspecific) monoclonal or polyclonal antibodies of the same class or subclass (isotype) as those used in the assays (e.g., IgG1, IgG 2a2, IgM, etc.) can be used as "blockers". The concentration of the "blockers" (normally 1-100, $\mu\text{g}/\mu\text{l}$) is important, in
20 order to maintain the proper sensitivity yet inhibit any unwanted interference by mutually occurring cross reactive proteins in human serum. In addition, the buffer system containing the "blockers" needs to be optimized. Preferred buffers are those based on weak organic acids, such as imidazole, HEPES, MOPS, TES, ADA, ACES, HEPES, PIPES, TRIS, and the like, at physiological pH
25 ranges. Somewhat less preferred buffers are inorganic buffers such as phosphate, borate or carbonate. Finally, known protease inhibitors should be added (normally at 0.01-10 microns/ml) to the buffer which contains the "blockers".

30 There are many solid phase immunoabsorbents which have been employed and which can be used in the present invention. Well-known immunoabsorbents include nitrocellulose, glass, polystyrene, polypropylene, dextran, nylon and other materials; tubes, beads, and microtiter plates formed from or coated with
35 such materials, and the like. The immobilized antibodies can be

either covalently or physically bound to the solid phase immunoadsorbent, by techniques such as covalent bonding via an amide or ester linkage, or by absorption. Those skilled in the art will know many other suitable solid phase immunoadsorbents and methods for immobilizing antibodies thereon, or will be able to ascertain such, using no more than routine experimentation.

Details of the operation and practice of the present invention are set forth in the specific examples which follow. However, these examples are not to be interpreted as limiting the scope of the present invention.

**EXAMPLE 1: Method of Making the Monoclonal Antibodies
Specific for PvESP-1 and PvESP-2**

Balb/c mice were immunized intraperitoneally with 5×10^8 purified *P. vivax* (of the Belem strain infected red blood cells (IRBC) in complete Freund's adjuvant. Immunization was repeated at 2 and 7 weeks using incomplete Freund's adjuvant and finally at 14 weeks without adjuvant. 3 days later, spleen cells from the immunized mouse were fused with myeloma cell line NY-FOX (Hyclone, Utah; Taggart, *Science*, 219:1228-1230, 1983) according to the basic method of Galfre et al. (*Nature*, 266:550-552, 1977). Cells were plated directly into microtiter wells and cultured (Renner et al., *Proc. Natl. Acad. Sci. USA*, 77:6797-6799, 1980) such that 1 to 2 weeks later, 1 or more hybrid colonies were observed in all wells. Culture supernatants were collected and screened by immunofluorescence assay using smears of *P. vivax* infected blood that also contained normal red blood cells. Those cells producing antibodies which selectively reacted with IRBCs were expanded and cryopreserved. Secondary screening was performed by SDS-PAGE with hybridoma culture supernatants from expanded cultures that had been obtained by centrifugation. Those mAbs which reacted with *P. vivax* blood stage extracts and culture supernatants (prepared essentially as described in Galinski et al., *Cell*, 69:1213-1226, 1992) were selected for

further study. Three such mAbs are designated 1D11.G10, 3D4.A2, and 1A3.B4.

5 **EXAMPLE 2: Screening of *P. vivax* λ ZAPII**
Expression Library with the mAbs

10 *P. vivax* genomic DNA was isolated and digested with mung bean nuclease (U.S. Biochemical) following the procedures of Vernick et al. (*Nucl. Acids Res.*, 16:6883-6896, 1988) and as modified by Galinski et al. (*supra*). Specifically, the DNA was digested with 42.5-45% formamide. The digested DNA was ligated into the λ ZAPII vector (Stratagene, LaJolla, CA) and the resulting phage were used to infect *E. coli*. Expression was induced by growth on IPTG (isopropylthio- β -D-galactoside) containing nitrocellulose plates overlaying the agar plates, and the resulting plaques were screened with the mAbs using standard immunodetection methods (see, for example, Maniatis, *Molecular Cloning: A Laboratory Manual*, 2nd ed., Chapter 12, 1989).

20 After screening approximately 3×10^5 recombinant plaques, mAb 1D11.G10 specifically recognized one recombinant phage plaque. This phage clone, PvMB3.3.1 was purified, and *in vivo* excised with the aid of helper phage R408 (Stratagene, LaJolla, CA) to yield the clone as a pBluescript plasmid retaining the recombinant DNA as a 3.34 kb insert (Short et al., *Nucleic Acids Res.*, 16:7583, 1988).

25 After screening approximately 4×10^5 plaques, mAb 3D4.E2 also revealed one phage plaque recognized by the antibody. This clone, PvMB2.5.1 was plaque purified and *in vivo* excised, as above, to yield a pBluescript plasmid containing the 3.7 kb DNA insert.

30 **EXAMPLE 3: Expression of the cloned**
proteins in *E. coli*

35 The isolated pBluescript plasmids were transformed into *E. coli* and expression was induced by growth in the presence of IPTG using standard methodology (Maniatis, *Molecular Cloning: A*

Laboratory Manual, 2nd ed., Chapter 1, 1989). Proteins produced by the cultures were isolated, separated on a gel, blotted and probed using standard techniques (Maniatis, *Molecular Cloning: A Laboratory Manual*, 2nd ed., Chapter 18, 1989). Probing the blot of PvMB3.3.1 with 1D11.G10 revealed multiple bands, the largest of which was 205-210 kD. Probing the blot of PvMB2.5.1 revealed a 60 kD band. These results indicate that the cloned inserts encode the epitopes recognized by these mAbs.

10 **EXAMPLE 4: Sequencing of the Pv3.3.1 insert**

The insert was directly sequenced from the pBluescript excision plasmid. Nested deletions of 100-300bp intervals were created using exonuclease III and mung bean nuclease (U.S. Biochemical, Cleveland, Ohio) and standard methodology. DNA sequences were generated using the dideoxy termination method sequencing methodology (Sanger et al., *Proc. Natl. Acad. Sci. USA*, 74:5463-5467, 1977). The entire PvESP-1 gene was sequenced on both DNA strands, and is SEQ ID No : 1. The deduced protein sequence (SEQ ID No : 2) was analyzed using Pustell and MacVector software programs (IBI). GenBank (release 70) and the Swiss Protein Data Bank (release 20) were screened for DNA and protein sequence homologies using the GCG Sequence Analysis Software Package, Version 7.0 (Genetics Computer Group, Inc.).

25 **EXAMPLE 5: Cross-reactivity Test with Other *Plasmodium* Species**

Figure 4A was produced as follows. *P. vivax* trophozoite-infected erythrocytes (2×10^4), 25 μ l of supernatants from *P. vivax* trophozoite and rupturing schizont-infected red blood cell cultures, and 20 μ l of a 1:10 dilution of *P. vivax* infected *Saimiri* monkey plasma were mixed with sample buffer and electrophoresed on an SDS-PAGE gel. The gel was electrophoretically transferred to 0.2 μ m nitrocellulose (NC) by Western blot (Towbin, H. et al., *Proc. Natl. Acad. Sci. USA*, 76:4350, 1979):

35 The NC was blocked with 3% non-fat dry milk and probed with mAb

1D11.G10 at 2 mg/ml in TBS. The blot was washed with TBS/0.05% tween 20 and reprobed with alkaline phosphatase conjugated anti-mouse IgG (Promega, Madison, Wisconsin) and developed with P-nitroblue tetrazolium chloride/5-bromo-4-chloro-3 indolyl phosphate (U.S. Biochemicals, Cleveland, Ohio).

Fig. 4B. was produced as follows. *P. vivax* IRBC were acquired from infected Saimiri monkey, *P. cynomolgi* (M strain) IRBC was from infected Rhesus monkeys, *P. knowlesi* IRBC were Saimiri monkey, *P. coatneyi* IRBC were from Rhesus monkeys, *P. falciparum* from human rbc in *in vitro* culture, *P. malariae* from infected Aotus monkeys, and *P. berghei* from infected rats. SDS-PAGE and nitrocellulose transfer were done as above with 1×10^5 parasites/lane dissolved in SDS-PAGE sample buffer. Indirect immunofluorescence assay was performed by making smears of IRBC on slides and reacting 1D11.G10 or 3D4.E2 with smears and using FITC conjugated goat anti-mouse IgG as secondary antibody with the same results as Western blot.

These results show that there is no cross-reactivity with other malarial species.

EXAMPLE 6: Diagnostic Assay using Alkaline Phosphatase and liposome conjugated mAb

Unlabelled mAb is absorbed to nitrocellulose sheets (5 μ m average pore size) at 5mg Ab/ml in PBS. The sheet is washed and blocked with 3% non-fat dry milk in TBS. The sheet is layered on an ELISA apparatus (Pierce) and a 96-well plexiglass top (like a slot blot apparatus) is secured in place over the nitrocellulose sheet. Diluted plasma (1:10-100 μ l) samples are applied to the wells and drawn through the nitrocellulose by vacuum. The wells are washed by vacuum and mAb 1D11.G10 conjugated to alkaline phosphatase is applied to the wells. Alkaline phosphatase conjugation was accomplished by the glutaraldehyde method of Avrameas (*Immunochemistry*, 6:43, 1967). The alkaline phosphatase conjugated mAb is pulled through the nitrocellulose by vacuum, the wells are washed, and then the developer sub-

strates NBT-BCIP are added to and pulled through the wells. Positive reactions are assessed by the appearance of a purple-violet to blue-black precipitate forming in the wells at the surface of the nitrocellulose. Eleven of the 15 plasma samples from *P. vivax* individuals were positive using the alkaline phosphatase conjugated mAb1D11.G10. All samples were assessed for infection with *P. vivax*, *P. falciparum*, or both, by Giemsa-stained thick films of blood samples. False positives thus, would show a positive reaction, but would be negative for *P. vivax* parasites in thick films. No such reactions were seen.

The liposome-based test was similar to the alkaline phosphatase-based test. As in the alkaline phosphatase assay, the secondary (reporter) mAb was conjugated to liposomes that contained a bright red to maroon dye. Thus, the appearance of red on the nitrocellulose was the reporter system and an enzymatic development step is not needed as in the alkaline phosphatase system. Thirteen of 15 infected samples were positive using the liposome conjugated 1D11.G10. This assay can also be adapted to a strip test where a mAb or polyclonal Ab is absorbed to a NC strip that overlays an absorbent pad. Then, test plasma, antibody conjugated liposomes, and washing solutions are wicked upwards by diffusion and a positive test is indicated by a red-to-magenta line across the NC strip assay.

EXAMPLE 7: Competitive Diagnostic Test for Malaria which Indicates Specific Infection with *P. vivax*

In a colorimetric immunoassay for PvESP-1 and/or PvESP-2, large, unilamellar phospholipid vesicles approximately 0.2 micrometers in diameter are loaded with high concentrations of Sulforhodamine B or a similar dye. The PvESP-1 and/or PvESP-2 is coupled to phosphatidylethanolamine or another component of the lipid vesicle, and incorporated into the lipid formulation, thus conferring immunological specificity. Methods of formation of the vesicles, loading the vesicles, and coupling the protein to

the phosphatidylethanolamine are disclosed in O'Connell et al. (*Clin. Chem*, 31:1424-1426). The liposomes are then used as tracers in simple competitive-binding immunoassays with antibody-coated tubes. The results are read spectrophotometrically.

- 5 Specific immunoassay methods are described in O'Connell et al., *supra*, as well as O'Connell, *MG and DI*, December, 1985, pp.31-36. As this is a competitive assay, the less signal seen, the more PvESP-1 and/or PvESP-2 will be present in the sample. It is anticipated that this assay will be selective for *P. vivax*
- 10 infection, given the selectivity of the antibodies 1D11.G10, 3D4.A2, and 1A3.B4 as shown in Example 5.

WE CLAIM:

1 1. An isolated purified peptide antigen comprising
2 all or part of the amino acid sequence of a species-specific
3 secreted blood-stage protein from *P. vivax* or fragment thereof,
4 said protein being present in detectable amounts in biological
5 samples of individuals infected with *P. vivax* malaria, said
6 antigen having the property of eliciting antibodies that recog-
7 nize said protein.

1 2. The peptide antigen of claim 1 wherein said blood
2 stage protein is PvESP-1.

1 3. The peptide antigen of claim 2, said antigen being
2 PvESP-1.

1 4. An isolated purified polypeptide comprising the
2 amino acid sequence of SEQ ID No. : 2 or immunogenic *P. vivax*
3 species-specific fragments thereof.

1 5. A DNA sequence selected from the group consisting
2 of (i) DNA which encodes all or part of the amino acid sequence
3 of SEQ ID No. : 2 said DNA encoding a peptide antigen according
4 to claim 1; and (ii) DNA hybridizing therewith under stringent
5 conditions.

1 6. The peptide antigen of claim 1 wherein said blood
2 stage protein is PvESP-2.

1 7. The peptide antigen of claim 6, said antigen being
2 PvESP-2.

1 8. Isolated and purified antibody immunochemically
2 reactive with a peptide antigen according to claim 1.

1 9. The antibody of claim 8 which is monoclonal.

1 10. Isolated and purified antibody immunochemically
2 reactive with a peptide antigen according to claim 2.

1 11. The antibody of claim 10 which is monoclonal.

1 12. Isolated and purified antibody immunochemically
2 reactive with a peptide antigen according to claim 6.

1 13. The antibody of claim 12 which is monoclonal.

1 14. A monoclonal antibody selected from the group
2 consisting of 1D11.G10 produced by the hybridoma ATCC accession
3 number _____; 3D4.E2 produced by the hybridoma ATCC accession
4 number _____; and 1A3.B4 produced by the hybridoma ATCC
5 accession number _____.

1 15. An assay for the selective identification of *P.*
2 *vivax* malarial infection in a susceptible mammal which comprises
3 the steps of:

4 (a) contacting a biological sample known to come
5 in contact with erythrocytes of said mammal with an antibody that
6 binds a *P. vivax* specific epitope of a species-specific secreted
7 blood-stage protein from *P. vivax* to form an antibody-antigen
8 complex;

9 (b) detecting said complex which indicates
10 whether said mammal is infected by *P. vivax*.

1 16. The assay of claim 14 wherein said antibody is
2 conjugated to a reporter substance.

1 17. The assay of claim 16, wherein said reporter
2 substance is selected from the group consisting of enzymatic

3 conjugates, dyes, radioisotopes, fluorescence, and particulate
4 labels.

5 18. The reporter substance of claim 17, wherein the
6 particulate label is selected from the group consisting of
7 liposome, latex, polystyrene, colloid metal and colloid nonmetal
8 labels.

1 19. The assay of claim 14 wherein said contacting step
2 is conducted in the co-presence of a known amount of labelled
3 peptide antigen comprising all or part of the sequence of said
4 secreted protein, said labelled antigen (i) also being recognized
5 by said antibody and (ii) competing with said secreted protein
6 for binding to said antibody; and said detecting step comprises
7 detecting said labelled antigen bound to said antibody or
8 detecting unbound labelled antigen.

1 20. The assay of claim 14 wherein said protein is
2 PvESP-1 or PvESP-2.

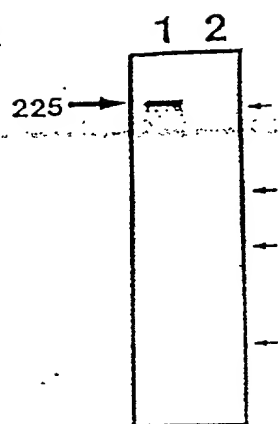
1 21. The assay of claim 14 wherein said protein and
2 said antigen are both PvESP-1 or are both PvESP-2.

ABSTRACT OF THE INVENTION

This invention is directed to novel species-specific *P. vivax* malarial peptide antigens which are proteins or fragments
5 of proteins secreted into the plasma of a susceptible mammalian host after infection, and to monoclonal or polyclonal antibodies directed against those antigens. The peptide antigens, monoclonal antibodies, and/or polyclonal antibodies are utilized in assays used to diagnose malaria, as well as to determine whether
10 *Plasmodium vivax* is the species responsible for the infection.

Figure 1.

A



B

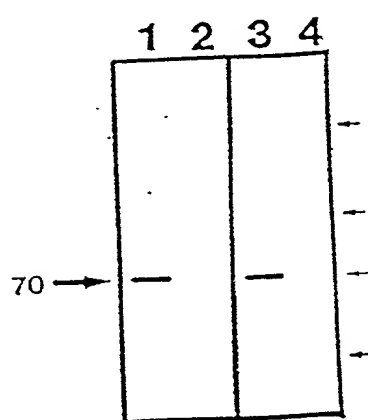


Figure 2.

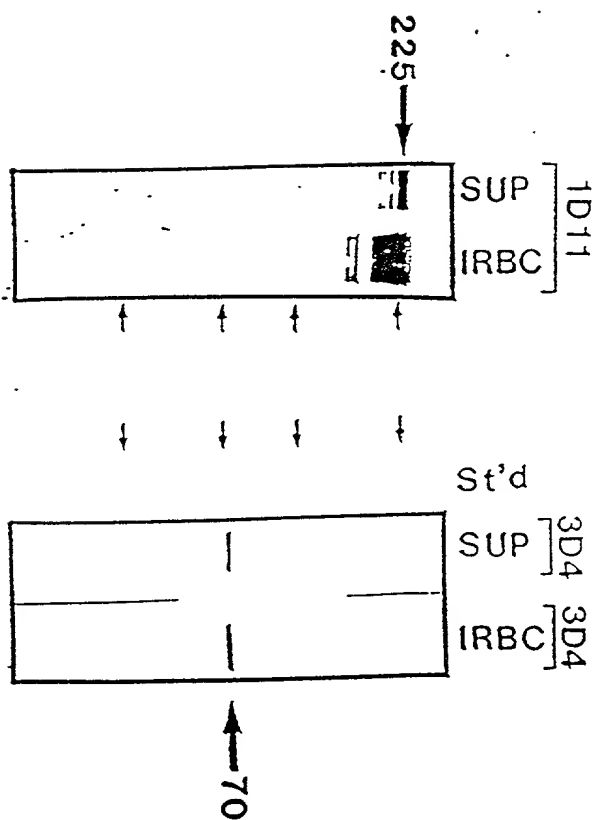
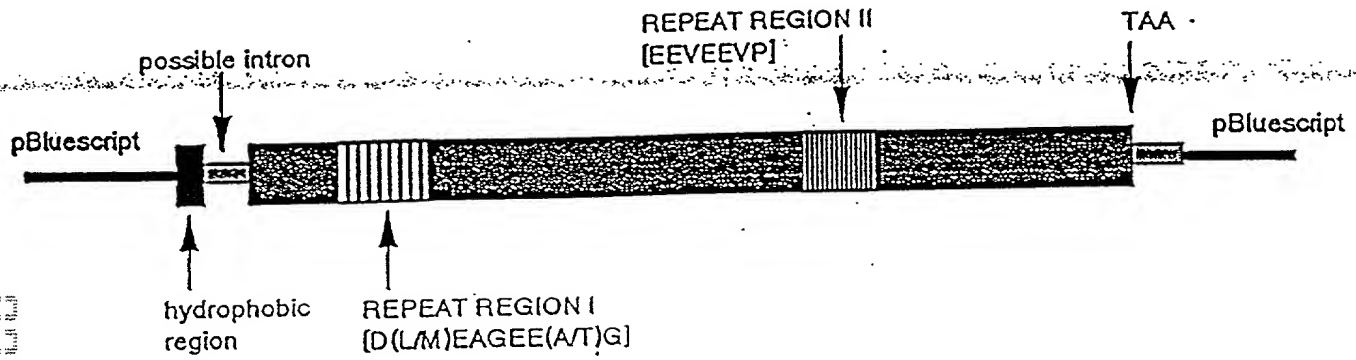
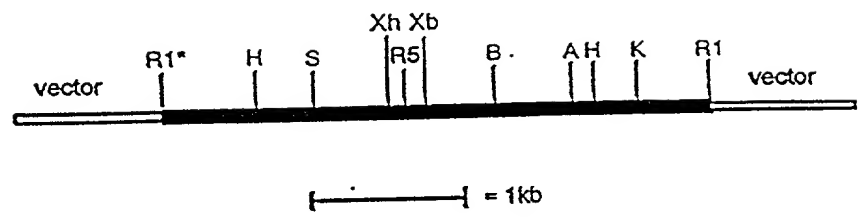


Figure 3.

A.

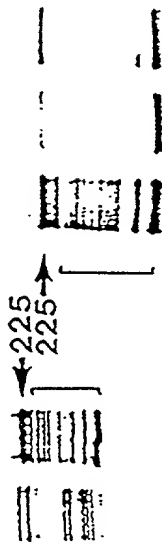


B.



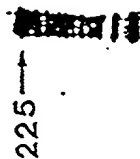
A

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15



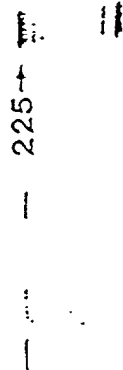
C

1 2 3 4 5 6 7 8 9 10 11



D

1 2 3 4 5 6 7



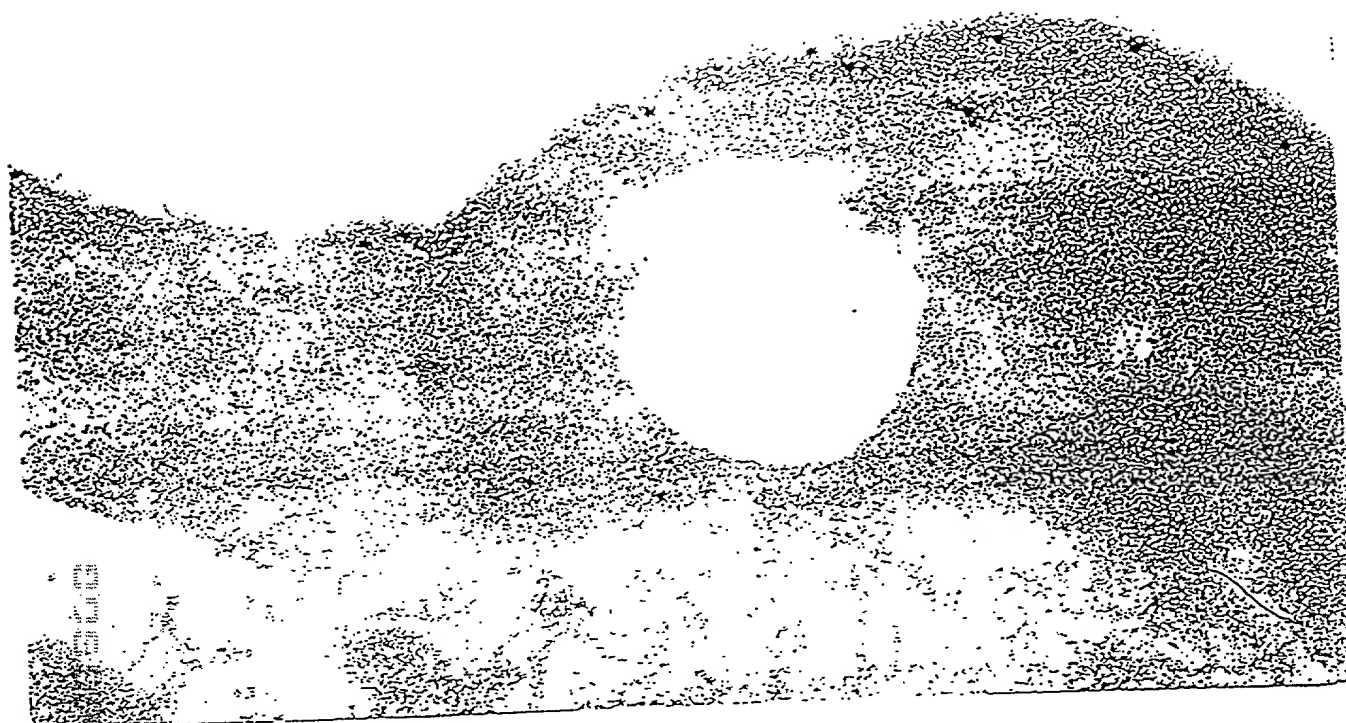
P. vivax ESP.i

T T G A T C A T A A A G A T T T C G A G C C A C T A T T C G A A C A A A T T G T G G C G G G T A C T G T A A A C A C T G T T A C G G G A C A A G A A T T G C C A A T G A A A C 990
 P D H I K I K D T F E G P C L F T E Q I V A G T A K H V T G G Q E L P M K
 C T G T A C C A T T A C C A G T G G C A G A A G C C C G C G A A G T A C C A G C G A A G A A T T A G A T G C C A C T C C A G G A G A C T G A C T T C C A T T A G A T G T T A 1080
 P V P L P V A E E P A Q V P A E E L D A T P E D D F A L D V
 C A G A A T C T C C G A G G A A G T A G A A T T A G T A T T A G A T G A A G A G G C A C T G A A G A A A T C A A C G G A A G T G G G A C C A C G G A A G A A G G A C C A A 1170
 T E S P E A E E V E L V L D E E A T E E E S T E V G G T A E G G P
 C C G A A G A A T T A G A T G C C A C T C C A G A G G A T G G A T T C G C A T T A G A C G A A A C T C G A A G A G A A A C G T A G A G G G A A G A A A 1260
 T E E L D A T P E D G G F R I R R N C R R R R N R R N V E G E E
 C A G A A G A A G C T G C A G A A G G A A G T A T C A G A A G A A A C T C C A G A A G G A A G A A G A G T T A G A G G C A A C T C C A G A G G A T G A T T T C G C A T T A G 1350
 T E E A A E G E V S E E T P E G E E E L E A T P E D D F A L
 A T G G A A C T A C A T T A G A A A A C C G A A G A A A C T G C A G A A G G A A G A A A C C G T A G A G G G A G A A A C C G T A G A G G G A G A A A C C G T A G 1440
 D G T T L E E T E E T A E G E E T V E G E E T V E G E E T V
 A G G G A A G A A G C T G C A G A A G G A A G A A G A G T T A G A G G C A A C T C C A G A G G A T G A C T T C C A A T T A G A A A C C A T C A G G A A G G A A G 1530
 E G E E A A E G E E E L E A T P E D D F Q L E E P S G E G E
 G G G A A G G A A G G A A G G G A A G G A A G G A A G C G T T A G T A G C A G T G C C A G T A G T G G C C G A A C C G G T A G A A G T A G T G A C T C C T G C T C 1620
 G E G E C A A G E G E G E G E A L V A V P V V A E P V E V V T P A
 A G C C T G T C A A C C A A T G G T C G C T C C A A C G G C A G A T G A A A C T T T A T T C G T T G A T A T C T T A G A T A C G A T T T A A C G T A T G C A G A C A T T A C A T 1710
 C P V K P M V A P T A D E T L F V D I L D N D L T Y A D I T
 C C T T T G A G C C A T T A T T T A A C A A A T C C T C A A G A T C C T G A T G C A G G A G A G C T G T A A C A G T A C C A T C A A A G G A A G C C C T G T A C A A G T A C 1800
 S F E P L F K Q I L K D P D A G E A V T V P S K E A P V Q V
 C A G T G G C A G T A G G G C C G C G A A G A A G T G C C A A C G G A A G A A T T G A T G C A A C T C C A A G A G G A C G A T T T C G A A T T A G A A G G A A C T G C A G A A G 1890
 P V A A G G P A Q E V E P T E E L M Q L Q E D D F E L E G T A E
 C T C A G A A G G A A T T A G T A T T A G A A G G A A G G A A C C A A C G G A A G A A G C C A A G A A G G A A G G A G C C A A C A G A A G G A A G T G C 1980
 A P E E G E L V L E G E G E P T E E E P R E G E P T E G E V
 C A G A A G A A A T T A G A G G C A A C T C C A G A G G A C G A T T T C G A A T T A G A A A C C A A C A G G A A G A A G T A G A A A C C G T A G A G G G C G A A G 2070
 P E E E T E A T P E D D F E L E E P T G E E V E E T V E G E
 A A A C T G C A A G G A A G A A G T G G A A G A G T A C T G C A A G T A G A A A G T G G A A G A G G T A C C T G C A A G T A G A A A G T G G A A G A G G 2160
 E T A A E G E E V E E V P A E V E E V E E V P A E V E E V E E
 T A C C A A G A A G T A G A A G A G T A C C C G C A G A A G T A G A A G A A G T G G A A G A G G T A C C A G A A A G T G G A A G A G G T A C C A G A A A G T G G A A G 2250
 V P E E V E E V P A E V E E V P E E V E E V P E E V E
 A G G T A C C A G A A A G T G G A A G A G G T A C C A G A A A G T G G A A G A A G T G G A A G A A G T A G A A A G T A G A A G T A C C A G G T A C C A G G T A G A A G T A G 2340
 E V P E E V E E V P E E V E E V E E V E E V E V P A V E V
 A A G T A C C A G C G G T A G T A G A A A G A G G T G C C A G A A A G T A G A A A A G A A A G A A G A A G A A C C A G T A G A G G A A A G A T G T A T T A C 2430
 E V P A V V E E E V P E E V E E E E E E E E E P V E E E D V L
 A A T T A G T A T A C C A T C G G A A G A T A T A C A A T T A G A C A A A C C A A G A A A G A C G A A T T A G G C T C T G G A A T T T T A T C T A T C A T C G A C A T G C 2520
 Q L V I P S E E D I Q L D K P K T G C A T G T L G S G I A L S I I D M
 A C T A C C A A G A C G T T C C A A G A A T T T A T G A A G A A G A A G A A A A C T G C A G T G A T T C C A T T G A A A C C G A A A G A T T T T G C A A A G G A A G A T T 2610
 H Y Q D V P K E F M E E E E E T A V Y P L K P E D F A K E D

[illegible]

3337

A



B

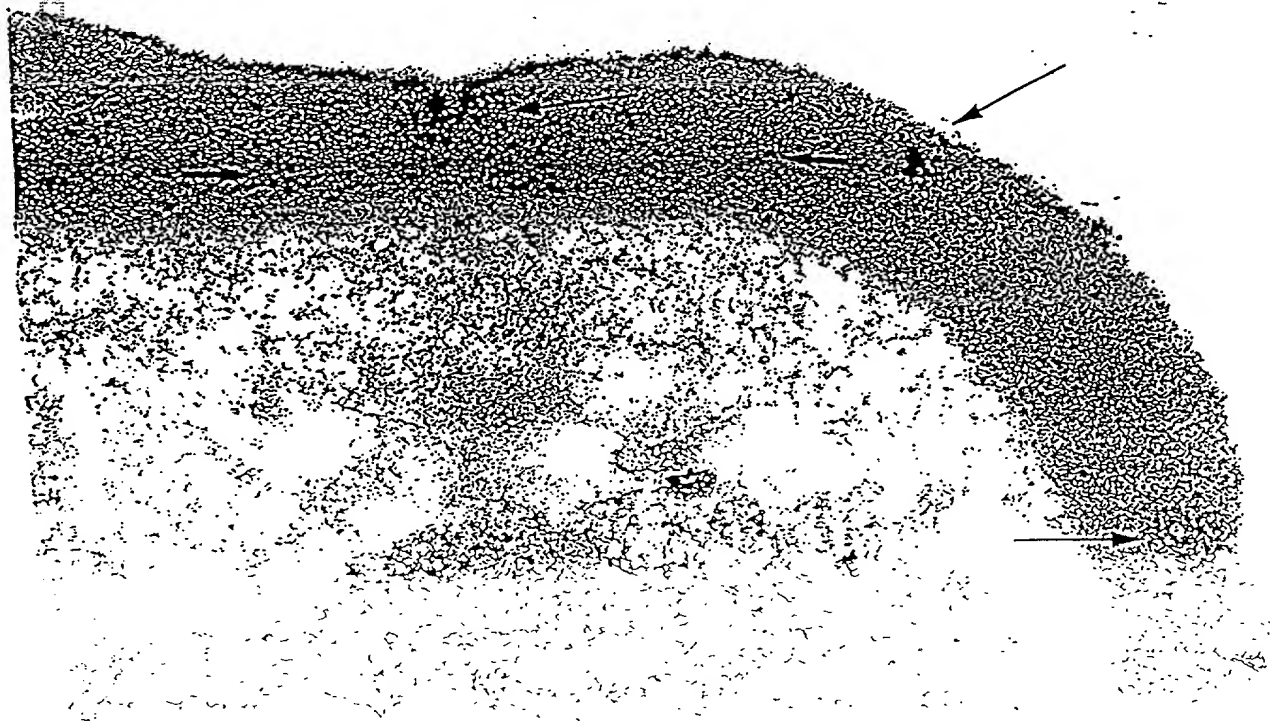
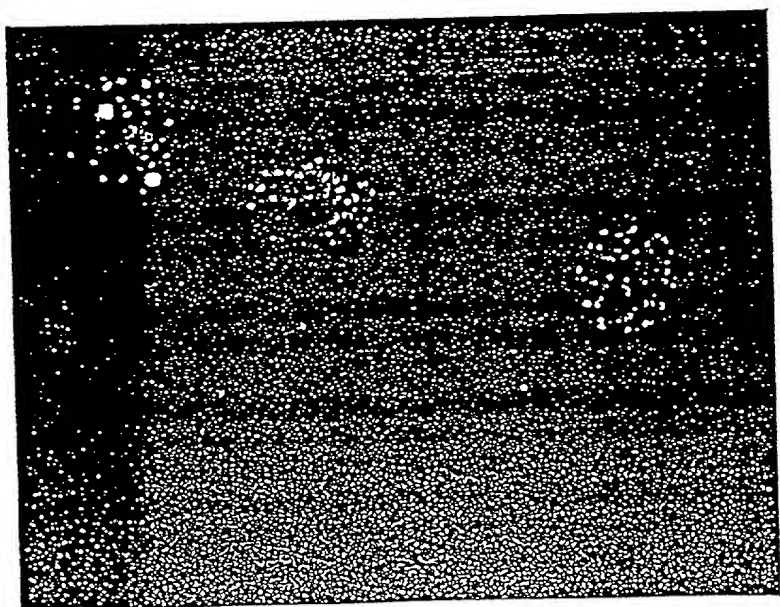


Figure 7.

A.



B.



DECLARATION
AND POWER OF ATTORNEY
Original Application

As a below named inventor, I declare that the information given herein is true, that I believe that I am the original, first and sole inventor if only one name is listed at 1 below, or a joint inventor if plural inventors are named below, of the invention entitled:

PLASMODIUM VIVAX BLOOD STAGE ANTIGENS, ANTIBODIES, AND
DIAGNOSTIC ASSAYS

which is described and claimed in:

☐ the attached specification or

☒ the specification in application Serial No. 08/072,610, filed June 2, 1993

(for declaration not accompanying appl.)

that I do not know and do not believe that the same was ever known or used in the United States of America before my or our invention thereof or patented or described in any printed publication in any country before my or our invention thereof, or more than one year prior to this application, or in public use or on sale in the United States of America more than one year prior to this application, that the invention has not been patented or made the subject of an inventor's certificate issued before the date of this application in any country foreign to the United States of America on an application filed by me or my legal representatives or assigns more than twelve months prior to this application, that I acknowledge my duty to disclose information of which I am aware which is material to the examination of this application in accordance with 37 CFR §1.56(b), and that no application for patent or inventor's certificate on this invention has been filed by me or my legal representatives or assigns in any country foreign to the United States of America except as identified below. I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

FOREIGN APPLICATION(S), IF ANY, FILED WITHIN 12 MONTHS
PRIOR TO THE FILING DATE OF THIS APPLICATION

<u>COUNTRY</u>	<u>APPLICATION NO.</u>	<u>DATE OF FILING</u>	<u>PRIORITY CLAIMED UNDER 35 U.S.C. §119</u>
----------------	------------------------	---------------------------	--

No

ALL FOREIGN APPLICATIONS, IF ANY, FILED MORE THAN 12 MONTHS
PRIOR TO THE FILING DATE OF THIS APPLICATION

<u>COUNTRY</u>	<u>APPLICATION NO.</u>	<u>DATE OF FILING</u>	<u>PRIORITY CLAIMED UNDER 35 U.S.C. §119</u>
----------------	------------------------	---------------------------	--

No

POWER OF ATTORNEY:

As a named inventor, I hereby appoint the following attorney(s) and/or agents(s) to prosecute this application and transact all business in the Patent and Trademark office connected therewith. Morris Relson #15,108, Gordon D. Coplein #19,165, William F. Dudine, Jr. #20,569, Michael J. Sweedler #19,937, S. Peter Ludwig #25,351, Paul Fields #20,298, Joseph B. Lerch #26,936, Melvin C. Garner #26,272, Ethan Horwitz #27,646, Beverly B. Goodwin #28,417, Adda C. Gogoris #29,714, Martin E. Goldstein #20,869, Bert J. Lewen #19,407, Henry Sternberg #22,408, Peter C. Schechter #31,662, Robert Schaffer #31,194, David R. Francescani #25,159

all of the firm of DARBY & DARBY P.C., 805 Third Avenue, New York, NY 10022

SEND CORRESPONDENCE TO:

DIRECT TELEPHONE CALLS TO:

DARBY & DARBY P.C.
805 Third Avenue
New York, NY 10022

212-527-7700

FULL NAME OF INVENTOR 1

LAST NAME: Barnwell FIRST NAME: John

MIDDLE NAME: W.

CITY: New York

STATE OR FOREIGN COUNTRY: New York

COUNTRY OF CITIZENSHIP: United States of America

POST OFFICE ADDRESS: 3 Washington Square Village

CITY: New York

STATE OR COUNTRY: New York, U.S.A.

ZIP CODE: 10012

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

SIGNATURE OF INVENTOR 1:

John W. Barnwell

DATED: 8/5/93

CERTIFICATE OF MAILING

I hereby certify that this paper and every paper referred to therein as being enclosed is being deposited with the U.S. Postal Service as first class mail, postage prepaid, in an envelope addressed to: Commissioner of Patents & Trademarks, Washington, DC 20231,

on _____ (Date of Deposit)

Date

Name

EXPRESS MAIL CERTIFICATE

Date 9/21/00 Label No. 5262 8222 74 6 US

I hereby certify that, on the date indicated above I deposited this paper or fee with the U.S. Postal Service & that it was addressed for delivery to the Commissioner of Patents & Trademarks, Washington D.C. 20231 by "Express Mail for Office to Addressee" service.

D Beck
Name (Print)

[Signature]
Signature

5986/17686-US5

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: John W. Barnwell

Serial No.: Concurrently Herewith Group Art Unit:

Filed: Concurrently Herewith Examiner:

For: **POLYNUCLEOTIDES ENCODING PLASMODIUM VIVAX BLOOD STAGE ANTIGENS**

September 21, 2000

Hon. Commissioner of
Patents and Trademarks
Washington, DC 20231

DECLARATION IN SUPPORT OF *IN RE HAWKINS* AMENDMENT

Sir:

In the Preliminary Amendment filed concurrently herewith on September 21, 2000 in the above-identified continuation patent application, Applicant has amended the specification to include a portion of the Southern *et al.*

publication, which has been incorporated by reference as of June 2, 1993, the effective filing date of the parent application. This Declaration is being made pursuant to MPEP §608.01. The amendatory material consists of the portion of the same material incorporated by reference in the present application.

Respectfully submitted,



Adda C. Gogoris
Reg. No. 29,714
Attorney for Applicant(s)

DARBY & DARBY P.C.
805 Third Avenue, 27th Flr.
New York, NY 10022
212-527-7700

::ODMA\WORLD\DOX\W\15986\17686\KRJ1868.W51

SEQUENCE LISTING

1) GENERAL INFORMATION:

- (i) APPLICANT: Barnwell, John
- (ii) TITLE OF INVENTION: Plasmodium vivax Blood Stage Antigens,
Monoclonal Antibodies, and Diagnostic Assays
- (iii) NUMBER OF SEQUENCES: 2
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Darby and Darby
 - (B) STREET: 805 Third Ave.
 - (C) CITY: New York
 - (D) STATE: New York
 - (E) COUNTRY: USA
 - (F) ZIP: 10022-7513
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: US
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (vii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Gogoris, Adda
 - (B) REGISTRATION NUMBER: 29,714
 - (C) REFERENCE/DOCKET NUMBER: 5986/07686
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (212)527-7700
 - (B) TELEFAX: (212)753-6237
 - (C) TELEX: 236687

2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 3337 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Plasmodium vivax

(vii) IMMEDIATE SOURCE:

(B) CLONE: PvMB3.3.1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GAATTCGGT AAAGTAACAA CTATGGTTC GTATCTATAT ATAACCTTAC TAATTTTATC	60
TTTTGCTTTT CTTTAAATTC ATGCTTCAAC AGTAAGATAA AAATAATCTA TAAAAACTGC	120
TATATATACA TATATATTCA TAAGTGGCAT TTGTGAATTG CGATCATTTA AATTACGTA	180
AAAACAATAT TGAAAAAAT TTTTTTTTTT TTTTTTTTTT TGTTCACAG AACGATTTAG	240
AATTGGAAAA TGCTTCTGAT GATGTTGTAG AGGTGGAGGA TCCTTCAAAC GACGGTTTAG	300
AATTAGAAGA GGAAAATTTT GATGAGAATT CAGGTGATGA TGAAACTCTT TTAGATGCTA	360
CCCCGAAGA TGACTTTGCC TTAACAGATT TGCCAATTGA AGACGATGAG GAAGTCAACG	420
AAACGTTAGA TGGAGGTGAA TCATTAGGAG AGGTTTCCAC TGAAGATATG GAAACAGAAG	480
ATGGCTCAAC AGATGATACG GAAACAGAAG AAGGACTACC TGGTGATATG GAAGGAGAAG	540
AAGAAGCTGG CGATATGGAA GCAGGGGAAG AAGCTGGTGA TTTGGAAGCA GGGGAAGAAA	600
CTGGCGATTT GGAAGCAGGG GAAGAACTG GCGATTTGGA AGCAGGGGAA GAAGCTGGTG	660
ATTTGGAAGC AGGGGAAGAA ACTGGCGATT TGGAAGCAGG GGAAGAACT GGAGATGCGG	720
AACTGAAGA AGGAGCAACT GGAGATGCGG AAAGTGAATA TGGAGCAACT GTGTATGTAG	780
ACACGAAGA TAGTTCAGCT GATGGAGCAG AAAAAGTACA TGTTCCTGCT CAAGAAAATG	840
TACAACCTGC CGATAGTAAT GATGCCCTCT TTGGAAGTAT TTTGGATAAA GATATAATTT	900
TGATCATAT TAAAGATTTT GAGCCACTAT TCGAACAAAT TGTGGCGGGT ACTGCTAAAC	960
ATGTTACGGG ACAAGAATTG CCAATGAAAC CTGTACCATT ACCAGTGGCA GAAGAGCCCG	1020
CGCAAGTACC AGCGGAAGAA TTAGATGCCA CTCCAGAGGA TGAATTCGCA TTAGATGTTA	1080
TAGAATCTCC CGAGGAAGTA GAATTAGTAT TAGATGAAGA GGCAACTGAA GAAGAATCAA	1140
TGGAAGTGGG ACCAACGGAA GAAGGACCAA CCGAAGAATT AGATGCCACT CCAGAGGATG	1200
EATTTGCGAT TAGACGAAAC TGCAGAAGGA GAAACAGAAG AAACGTAGAG GGAGAAGAAA	1260
TAGAAGAAGC TGCAGAAGGA GAAGTATCAG AAGAACTCC AGAAGGAGAA GAAGAGTTAG	1320

AGGCAACTCC	AGAGGATGAT	TTCGCATTAG	ATGGAACTAC	ATTAGAAGAA	ACCGAAGAAA	1380
CTGCAGAAGG	AGAAGAAACC	GTAGAGGGAG	AAGAAACCGT	AGAGGGAGAA	GAAACCGTAG	1440
AGGGAGAAGA	AGCTGCAGAA	GGAGAAGAAG	AGTTAGAGGC	AACTCCAGAG	GATGACTTCC	1500
AATTAGAAGA	ACCATCAGGA	GAAGGAGAAG	GGGAAGGAGA	AGGAGAAGGG	GAAGGAGAAG	1560
GAGAAGCGTT	AGTAGCAGTG	CCAGTAGTGG	CCGAACCGGT	AGAAGTAGTG	ACTCCTGCTC	1620
AGCCTGTCAA	ACCAATGGTC	GCTCCAACGG	CAGATGAAAC	TTTATTCGTT	GATATCTTAG	1680
ATAACGATTT	AACGTATGCA	GACATTACAT	CCTTTGAGCC	ATTATTTAAA	CAAATCCTCA	1740
AGGATCCTGA	TGCAGGAGAG	GCTGTAACAG	TACCATCAAA	GGAAGCACCT	GTACAAGTAC	1800
CAGTGGCAGT	AGGGCCCGCG	CAAGAAGTGC	CAACGGAAGA	ATTGATGCAA	CTCCAAGAGG	1860
ACGATTTCGA	ATTAGAAGGA	ACTGCAGAAG	CTCCAGAGGA	AGGAGAATTA	GTATTAGAAG	1920
GAGAAGGAGA	ACCAACGGAA	GAAGAGCCAA	GAGAAGGAGA	GCCAACAGAA	GGAGAAGTGC	1980
CAGAGAAGA	ATTAGAGGCA	ACTCCAGAGG	ACGATTTCGA	ATTAGAAGAA	CCAACAGGAG	2040
AAGAAGTAGA	AGAAACCGTA	GAGGGCGAAG	AAACTGCAGA	AGGAGAAGAA	GTGGAAGAGG	2100
TACCTGCAGA	AGTAGAAGAA	GTGGAAGAGG	TACCTGCAGA	AGTAGAAGAA	GTGGAAGAGG	2160
TACCAGAAGA	AGTAGAAGAG	GTACCCGCAG	AAGTAGAAGA	AGTGGAAGAG	GTACCAGAAG	2220
AAGTGGAAGA	GGTACCAGAA	GAAGTGGAAG	AGGTACCAGA	AGAAGTGGA	GAGGTACCAG	2280
AAGAAGTGGA	AGAAGTGGA	GAAGTAGAAG	AAGTAGAGGT	ACCAGCGGTA	GTAGAAGTAG	2340
AAGTACCAGC	GGTAGTAGAA	GAAGAGGTGC	CAGAAGAAGT	AGAAGAAGAA	GAAGAAGAGG	2400
AAGTACCAGT	AGAGGAAGAA	GATGTATTAC	AATTAGTAAT	ACCATCGGAA	GAAGATATAC	2460
AATTAGACAA	ACCAAAGAAA	GACGAATTAG	GCTCTGGAAT	TTTATCTATC	ATCGACATGC	2520
ACTACCAAGA	CGTTCCAAAG	GAATTTATGG	AAGAAGAAGA	AGAAACTGCA	GTGTATCCAT	2580
TGAAACCAGA	AGATTTTGCA	AAGGAAGATT	CACAATCTAC	AGAATGGCTC	ACATTCATTC	2640
AAGGCCTAGA	AGGCGACTGG	GAACGATTAG	AAGTGAGCTT	AAATAAGGCT	AGAGAAAGAT	2700
GGATGGAACA	AAGAAATAAA	GAATGGGCTG	GCTGGCTTCG	CTTAATTGAA	AATAAATGGT	2760
CAGAATATAG	TCAAATTTCA	ACAAAAGGAA	AGGACCCAGC	TGGTTTGAGA	AAACGAGAGT	2820
GGAGCGACGA	GAAATGGAAA	AAATGGTTTA	AAGCAGAAGT	CAAATCCCAA	ATTGATTAC	2880
ACTTGAAAAA	ATGGATGAAC	GACACTCATT	CCAATTTATT	TAAAATTCTT	GTGAAAGATA	2940

TGTCAACAATT TGAAAACAAG AAAACCAAAG AATGGTTAAT GAATCACTGG AAAAAGAACG 3000
 AACGGGGTTA TGGTTCTGAA TCATTTGAAG TTATGACCAC ATCAAAATTA TTAAATGTGG 3060
 CTAAGAGTCG AGAATGGTAC CGTGCCAATC CTAATATAAA TAGAGAAAGA AGAGAACTCA 3120
 TGAAATGGTT TCTCCTAAAA GAAAACGAAT ATTTAGGACA AAGAATGGAA AAAATGGACT 3180
 CATTGGAAAA AAGTTAAATT TTTTGTGTTC AATTCAATGT GTACAACATT TTCTGGAAAA 3240
 CGCCTAACCA AGGAAGAATG GAATCAATTT GTTAATGAAA TAAAAGTTTG AATTATAGAA 3300
 AAAAGAACAG ATTATTCTCT TATAAAATAA ATAATTC 3337

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1018 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (iii) HYPOTHETICAL: YES
- (iv) ANTI-SENSE: NO
- (v) FRAGMENT TYPE: C-terminal
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: *Plasmodium vivax*
- (vii) IMMEDIATE SOURCE:
 - (B) CLONE: PvMB3.3.1
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Asn	Ser	Gly	Lys	Val	Thr	Thr	Met	Val	Ser	Tyr	Leu	Tyr	Ile	Thr	Leu	1	5	10	15
Leu	Ile	Leu	Ser	Phe	Ala	Phe	Leu	Leu	Ile	His	Ala	Ser	Thr	Asn	Asp	20	25	30	
Leu	Glu	Leu	Glu	Asn	Ala	Ser	Asp	Asp	Val	Val	Glu	Val	Glu	Asp	Pro	35	40	45	
Ser	Asn	Asp	Gly	Leu	Glu	Leu	Glu	Glu	Glu	Asn	Phe	Asp	Glu	Asn	Ser	50	55	60	
Gly	Asp	Asp	Glu	Thr	Leu	Leu	Asp	Ala	Thr	Pro	Glu	Asp	Asp	Phe	Ala	65	70	75	80
Leu	Thr	Asp	Leu	Pro	Ile	Glu	Asp	Asp	Glu	Glu	Val	Asn	Glu	Thr	Leu				

85

90

95

Asp	Gly	Gly	Glu	Ser	Leu	Gly	Glu	Val	Ser	Thr	Glu	Asp	Met	Glu	Thr	
			100					105					110			
Glu	Asp	Gly	Ser	Thr	Asp	Asp	Thr	Glu	Thr	Glu	Glu	Gly	Leu	Pro	Gly	
		115					120					125				
Asp	Met	Glu	Gly	Glu	Glu	Glu	Ala	Gly	Asp	Met	Glu	Ala	Gly	Glu	Glu	
	130					135					140					
Ala	Gly	Asp	Leu	Glu	Ala	Gly	Glu	Glu	Thr	Gly	Asp	Leu	Glu	Ala	Gly	
	145				150					155						160
Glu	Glu	Thr	Gly	Asp	Leu	Glu	Ala	Gly	Glu	Glu	Ala	Gly	Asp	Leu	Glu	
				165					170						175	
Ala	Gly	Glu	Glu	Thr	Gly	Asp	Leu	Glu	Ala	Gly	Glu	Glu	Thr	Gly	Asp	
				180				185						190		
Ala	Glu	Thr	Glu	Glu	Gly	Ala	Thr	Gly	Asp	Ala	Glu	Thr	Glu	Asn	Gly	
				195			200					205				
Ala	Thr	Val	Tyr	Val	Asp	Thr	Glu	Asp	Ser	Ser	Ala	Asp	Gly	Ala	Glu	
	210					215					220					
Lys	Val	His	Val	Pro	Ala	Gln	Glu	Asn	Val	Gln	Pro	Ala	Asp	Ser	Asn	
	225				230					235					240	
Asp	Ala	Leu	Phe	Gly	Ser	Ile	Leu	Asp	Lys	Asp	Ile	Ile	Phe	Asp	His	
				245					250					255		
Ile	Lys	Asp	Phe	Glu	Pro	Leu	Phe	Glu	Gln	Ile	Val	Ala	Gly	Thr	Ala	
			260					265					270			
Lys	His	Val	Thr	Gly	Gln	Glu	Leu	Pro	Met	Lys	Pro	Val	Pro	Leu	Pro	
		275					280					285				
Val	Ala	Glu	Glu	Pro	Ala	Gln	Val	Pro	Ala	Glu	Glu	Leu	Asp	Ala	Thr	
	290					295					300					
Pro	Glu	Asp	Asp	Phe	Ala	Leu	Asp	Val	Thr	Glu	Ser	Pro	Glu	Glu	Val	
	305				310					315					320	
Glu	Leu	Val	Leu	Asp	Glu	Glu	Ala	Thr	Glu	Glu	Glu	Ser	Thr	Glu	Val	
				325					330					335		
Gly	Pro	Thr	Glu	Glu	Gly	Pro	Thr	Glu	Glu	Leu	Asp	Ala	Thr	Pro	Glu	
			340					345					350			
Asp	Gly	Phe	Arg	Ile	Arg	Arg	Asn	Cys	Arg	Arg	Arg	Asn	Arg	Arg	Asn	
		355					360					365				
Val	Glu	Gly	Glu	Glu	Thr	Glu	Glu	Ala	Ala	Glu	Gly	Glu	Val	Ser	Glu	

370

375

380

Glu Thr Pro Glu Gly Glu Glu Glu Leu Glu Ala Thr Pro Glu Asp Asp
385 390 395 400

Phe Ala Leu Asp Gly Thr Thr Leu Glu Glu Thr Glu Glu Thr Ala Glu
405 410 415

Gly Glu Glu Thr Val Glu Gly Glu Glu Thr Val Glu Gly Glu Glu Thr
420 425 430

Val Glu Gly Glu Glu Ala Ala Glu Gly Glu Glu Glu Leu Glu Ala Thr
435 440 445

Pro Glu Asp Asp Phe Gln Leu Glu Glu Pro Ser Gly Glu Gly Glu Gly
450 455 460

Glu Gly Glu Gly Glu Gly Glu Gly Glu Gly Glu Ala Leu Val Ala Val
465 470 475 480

Pro Val Val Ala Glu Pro Val Glu Val Val Thr Pro Ala Gln Pro Val
485 490 495

Lys Pro Met Val Ala Pro Thr Ala Asp Glu Thr Leu Phe Val Asp Ile
500 505 510

Leu Asp Asn Asp Leu Thr Tyr Ala Asp Ile Thr Ser Phe Glu Pro Leu
515 520 525

Phe Lys Gln Ile Leu Lys Asp Pro Asp Ala Gly Glu Ala Val Thr Val
530 535 540

Pro Ser Lys Glu Ala Pro Val Gln Val Pro Val Ala Val Gly Pro Ala
545 550 555 560

Gln Glu Val Pro Thr Glu Glu Leu Met Gln Leu Gln Glu Asp Asp Phe
565 570 575

Glu Leu Glu Gly Thr Ala Glu Ala Pro Glu Glu Gly Glu Leu Val Leu
580 585 590

Glu Gly Glu Gly Glu Pro Thr Glu Glu Glu Pro Arg Glu Gly Glu Pro
595 600 605

Thr Glu Gly Glu Val Pro Glu Glu Glu Leu Glu Ala Thr Pro Glu Asp
610 615 620

Asp Phe Glu Leu Glu Glu Pro Thr Gly Glu Glu Val Glu Glu Thr Val
625 630 635 640

Glu Gly Glu Glu Thr Ala Glu Gly Glu Glu Val Glu Glu Val Pro Ala
645 650 655

Glu Val Glu Glu Val Glu Glu Val Pro Ala Glu Val Glu Glu Val Glu

660					665					670									
Glu	Val	Pro	Glu	Glu	Val	Glu	Glu	Val	Pro	Ala	Glu	Val	Glu	Glu	Val				
675					680					685									
Glu	Glu	Val	Pro	Glu	Glu	Val	Glu	Glu	Val	Pro	Glu	Glu	Val	Glu	Glu				
690					695					700									
Val	Pro	Glu	Glu	Val	Glu	Glu	Val	Pro	Glu	Glu	Val	Glu	Glu	Val	Glu				
705					710					715					720				
Glu	Val	Glu	Glu	Val	Glu	Val	Pro	Ala	Val	Val	Glu	Val	Glu	Val	Pro				
725					730					735									
Ala	Val	Val	Glu	Glu	Glu	Val	Pro	Glu	Glu	Val	Glu	Glu	Glu	Glu	Glu				
740					745					750									
Glu	Glu	Glu	Pro	Val	Glu	Glu	Glu	Asp	Val	Leu	Gln	Leu	Val	Ile	Pro				
755					760					765									
Ser	Glu	Glu	Asp	Ile	Gln	Leu	Asp	Lys	Pro	Lys	Lys	Asp	Glu	Leu	Gly				
770					775					780									
Ser	Gly	Ile	Leu	Ser	Ile	Ile	Asp	Met	His	Tyr	Gln	Asp	Val	Pro	Lys				
785					790					795					800				
Glu	Phe	Met	Glu	Glu	Glu	Glu	Glu	Thr	Ala	Val	Tyr	Pro	Leu	Lys	Pro				
805					810					815									
Glu	Asp	Phe	Ala	Lys	Glu	Asp	Ser	Gln	Ser	Thr	Glu	Trp	Leu	Thr	Phe				
820					825					830									
Ile	Gln	Gly	Leu	Glu	Gly	Asp	Trp	Glu	Arg	Leu	Glu	Val	Ser	Leu	Asn				
835					840					845									
Lys	Ala	Arg	Glu	Arg	Trp	Met	Glu	Gln	Arg	Asn	Lys	Glu	Trp	Ala	Gly				
850					855					860									
Trp	Leu	Arg	Leu	Ile	Glu	Asn	Lys	Trp	Ser	Glu	Tyr	Ser	Gln	Ile	Ser				
865					870					875					880				
Thr	Lys	Gly	Lys	Asp	Pro	Ala	Gly	Leu	Arg	Lys	Arg	Glu	Trp	Ser	Asp				
885					890					895									
Glu	Lys	Trp	Lys	Lys	Trp	Phe	Lys	Ala	Glu	Val	Lys	Ser	Gln	Ile	Asp				
900					905					910									
Ser	His	Leu	Lys	Lys	Trp	Met	Asn	Asp	Thr	His	Ser	Asn	Leu	Phe	Lys				
915					920					925									
Ile	Leu	Val	Lys	Asp	Met	Ser	Gln	Phe	Glu	Asn	Lys	Lys	Thr	Lys	Glu				
930					935					940									
Trp	Leu	Met	Asn	His	Trp	Lys	Lys	Asn	Glu	Arg	Gly	Tyr	Gly	Ser	Glu				

